UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS

Departamento de Bioquímica y Biología Molecular I

TESIS DOCTORAL

Las ribotoxinas fúngicas como herramientas biotecnológicas

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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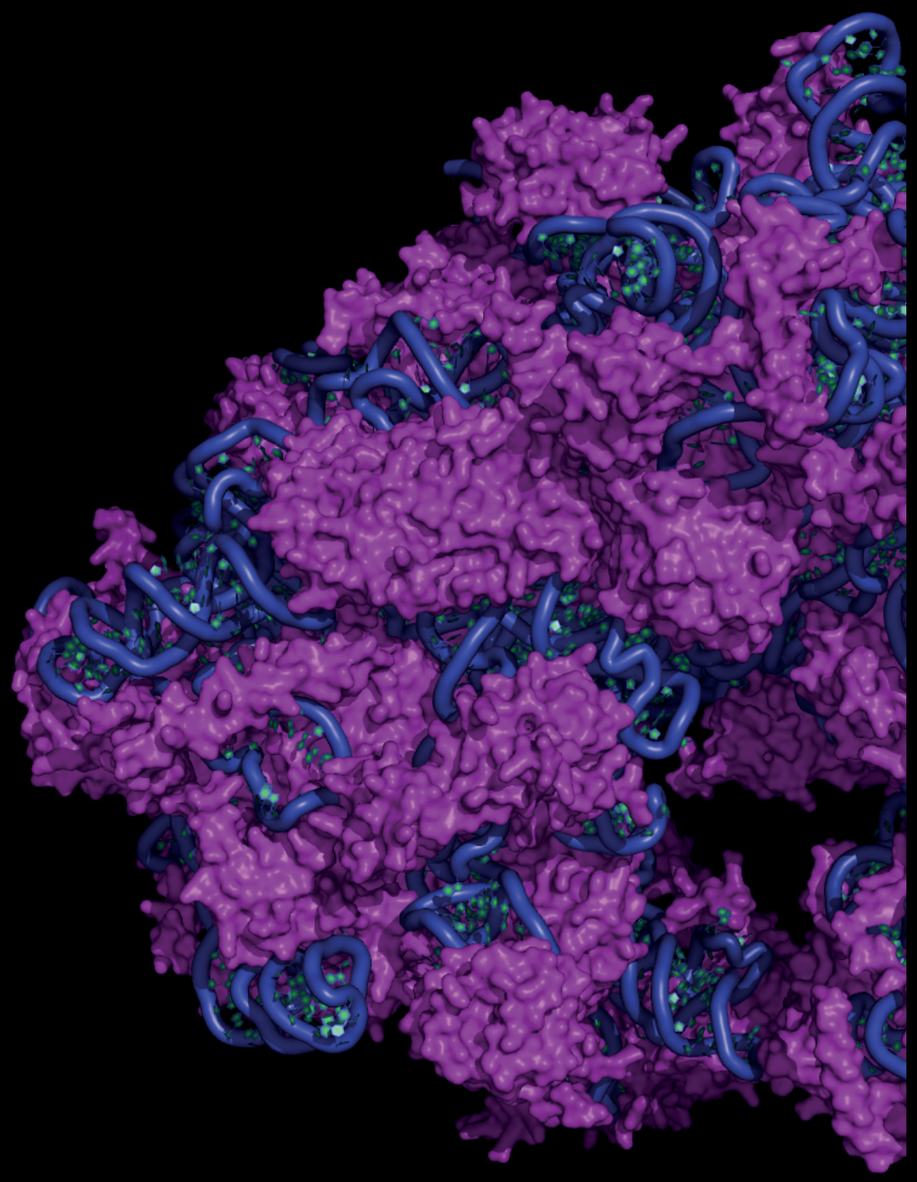

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MIRIAM OLOMBRADA SACRISTÁN

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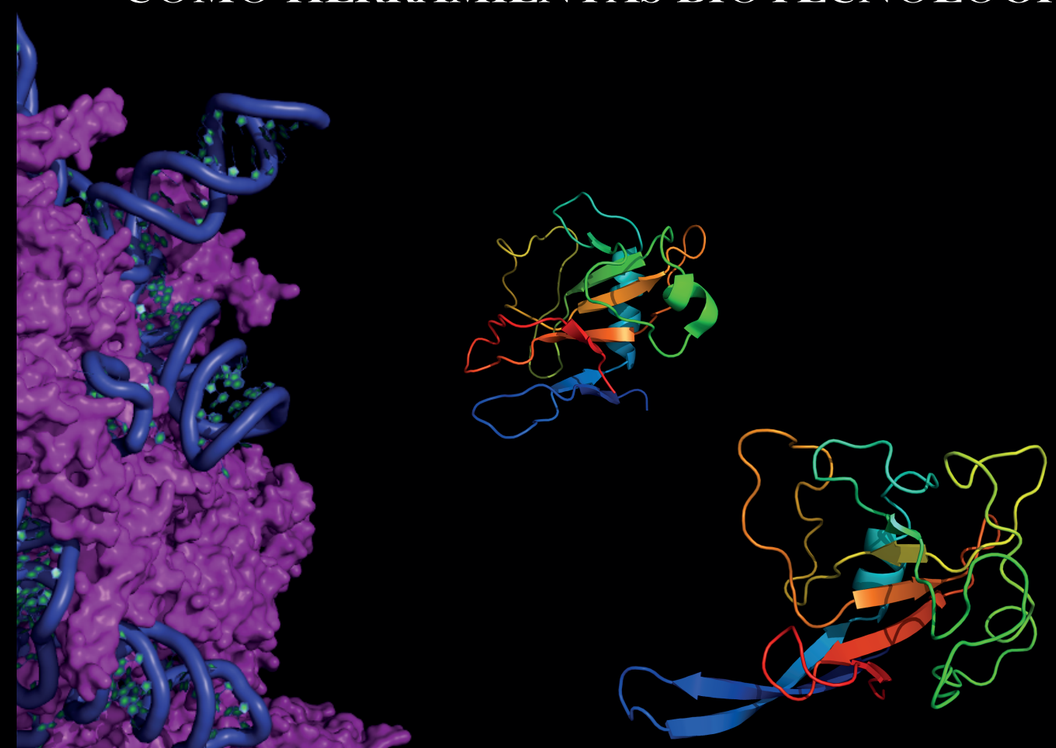

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"You are capable of more than you know. Choose a goal that seems right for you and strive to be the best, however hard the path. Aim high. Behave honorably. Prepare to be alone at times, and to endure failure. Persist! The world needs all you can give"

- Edward O. Wilson

A mis padres

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ABREVIATURAS/ABBREVIATIONS

A	Adenina
ANTS	Ácido 8-aminonaftaleno-1,3,6-trisulfónico
C	Citosina
CD	Dicroísmo circular
cDNA	Ácido desoxirribonucleico (ADN) codificante
DBA	Anemia de Diamond-Blackfan (*Diamond-Blackfan Anemia*)
DDT	dichlorodiphenyltrichloroethane
DMPG	Dimiristoilfosfatidilglicerol
DPX	N,N-p-xilene-byspiridiniumbromide
G	Guanina
GAC	Centro de activación de la GTPasa
GM/OMG	Organismo Modificado Genéticamente (*Genetically Modified Organism*)
GRAS	Generalmente considerada segura (*Generally Regarded As Safe*)
HtA	Hirsutelina A
IMTX	Inmunotoxina
PAGE	Electroforesis en geles de poliacrilamida
PBS	Tampón fosfato salino
PCR	Reacción en cadena de la polimerasa
PDB	Protein Data Bank
PPE	Poison Primer Extension
PolyPhe	Polifenilalanina
RIP	Proteína inactivante del ribosoma (*Ribosome-Inactivating Protein*)
RNase	Ribonucleasa
RP	Proteína ribosomal (*Ribosomal Protein*)
rRNA	Ácido ribonucléico ribosomal

SDS	Dodecil sulfato sódico
SSD	Síndrome de Shwachman-Diamond (*Shwachman-Diamond Syndrome*)
SRL	Lazo sarcina/ricina (*Sarcin-Ricin Loop*)
T	Timina
TCA	Ácido tricloroacético
TLC	Cromatografía en capa fina (*Thin layer chromatography*)
U	Uracilo
UV	Ultravioleta

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Introducción

El establecimiento de interacciones biológicas entre organismos conlleva en multitud de ocasiones la producción de toxinas de todo tipo. De entre todas estas toxinas destacan, por su potencia, las ribotoxinas. Esta familia de ribonucleasas fúngicas se ha estudiado con detalle desde su descubrimiento a principios de los años 60. Las primeras que se describieron, como la **α-sarcina**,están producidas por hongos del género *Aspergillus,* aunque también se han encontrado en otras especies como el hongo entomopatógeno *Hirsutella thompsonii* que produce la **hirsutelina A** (HtA).

La estructura tridimensional de varias de estas ribotoxinas se conoce con resolución atómica, y el análisis mutacional ha permitido asignar a diferentes residuos papeles concretos, como su implicación en la catálisis, el reconocimiento del ribosoma o la interacción con membranas. Su elevada especificidad reside en su capacidad para hidrolizar un único enlace fosfodiester de una estructura conservada de rRNA que se conoce como el lazo de sarcina-ricina (SRL) y que, localizado en la subunidad mayor del ribosoma, juega un papel esencial durante la traducción. El corte de este enlace inhibe la biosíntesis de proteínas y produce la muerte celular. Además, la toxicidad de las ribotoxinas también depende de su habilidad para cruzar membranas, cuya composición lipídica es crítica a la hora de determinar su especificidad citotóxica, siendo más eficientes en células transformadas o infectadas por virus. Debe haber, además, una serie de interacciones específicas con elementos del ribosoma que las guíen hacia el SRL, algunas de las cuales ya se han propuesto como resultado de trabajos anteriores.

A pesar de que la función biológica de las ribotoxinas no se ha esclarecido aún, hay estudios que han sugerido propiedades insecticidas, apoyando su participación en mecanismos de defensa y parasitismo. El estudio detallado de la estructura y función de esta familia de proteínas tóxicas ha mostrado su potencial uso como herramientas biotecnológicas. Se ha avanzado mucho en su uso como agentes antitumorales formando parte de inmunotoxinas, y se ha estudiado en detalle su implicación en alergias e infecciones por *Aspergillus*, por ejemplo. En esta Tesis Doctoral se plantea también su uso como biopesticidas en el control de plagas, y se ha comenzado a explorar la posibilidad de utilizarlas como herramientas específicas en el estudio de la biogénesis del ribosoma, facilitando con ellas una mayor comprensión de enfermedades humanas relacionadas con el ribosoma, las llamadas ribosomopatías.

Objetivos

-Estudio de las relaciones estructura-función en las ribotoxinas. En concreto, en lo referente al papel que juegan en la actividad citotóxica algunos residuos de los bucles 2 y 3 de la α-sarcina, así como el papel de la horquilla β-amino-terminal y algunos residuos del bucle 5 de la HtA.

- Caracterización de una nueva ribotoxina, denominada anisoplina, semejante a HtA, y producida por el hongo *Metarhizium anisopliae*.

-Análisis comparativo de la función insecticida de las ribotoxinas fúngicas más representativas, y evaluación de su potencial uso en el control de plagas, como alternativa a los pesticidas tradicionales.

-Estudio de la interacción de las ribotoxinas fúngicas con el ribosoma eucariota, utilizando como modelo la levadura *Saccharomyces cerevisiae*, analizando la participación del tallo ribosómico eucariota en el mecanismo de interacción de la ribotoxina α-sarcina con el ribosoma.

-Determinación del efecto de las ribotoxinas fúngicas sobre el proceso de biogénesis del ribosoma eucariota.

Resultados y discusión

Relaciones estructura-función en las ribotoxinas

En primer lugar se estudiaron aspectos específicos de la estructura y la función de las ribotoxinas α-sarcina y HtA. En concreto, el papel que juegan los bucles 2 y 3 de la α-sarcina (resultados A1) y el bucle 5 y el extremo amino terminal de la HtA (resultados A2).

Los mutantes del bucle 3 de la α-sarcina estudiados permitieron demostrar que esta región es imprescindible para su actividad catalítica. K111 y K114 participan también en el acercamiento de las vesículas lipídicas, facilitando su agregación. Además, se ha descrito la existencia de una red de interacciones entre la K114 y la Y48, un residuo del centro activo clave en la especificidad de la ribotoxina por su sustrato. En cuanto al bucle 2, la H82 no parece ser esencial, si bien su sustitución por glutamina (H82Q) aumenta su capacidad de permeabilización de vesículas lipídicas.

La sustitución de los residuos 79 a 93 del bucle 2 en la α-sarcina por los correspondientes residuos de HtA (ADAI) acorta notablemente su extensión, afectando a la disposición geométrica del centro activo provocando la pérdida completa de actividad enzimática. Esta deleción, sin embargo, no cambia la funcionalidad frente a vesículas lipídicas, por lo que se puede deducir que esta secuencia no está involucrada en la interacción con membranas.

La horquilla β amino terminal de la HtA es significativamente más corta y posee menos cargas positivas que la de la α-sarcina. Ante la posibilidad de que el acortamiento de esta región se vea compensado por la mayor longitud y orientación del bucle 5, se han producido y caracterizado diferentes variantes mutantes de HtA. La deleción de la horquilla β amino terminal da lugar en este caso a una variante que mantiene la especificidad ribonucleolítica frente a ribosomas y SRL, pero con menor actividad que la HtA silvestre. Lo mismo ocurre con el resto de mutaciones puntuales

del bucle 5. Estos resultados sugieren que variaciones en el microentorno de los centros activos afectan de distinta manera a ambas ribotoxinas, presentando HtA una mayor plasticidad, ya que los cambios realizados pueden ser compensados sin que haya una pérdida de actividad. La α-sarcina poseería un centro activo más rígido, donde la modificación de los residuos implicados en la catálisis da lugar a variantes completamente inactivas.

El análisis del genoma del hongo entomopatógeno *Metarhizium anisopliae* reveló la presencia de una proteína que compartía un 70% de identidad de secuencia con HtA. Una proteína que resultó ser una nueva ribotoxina, denominada **anisoplina** (Anp) (resultados A3). La Anp produjo de forma recombinante y se caracterizó estructural y funcionalmente, demostrando que poseía la actividad ribonucleolítica específica característica de las ribotoxinas. Hasta ahora se pensaba que, por su menor tamaño, HtA era una excepción dentro de la familia de las ribotoxinas. La descripción de la Anp como una auténtica ribotoxina sugiere la posibilidad de que exista un subgrupo de proteínas de este tipo dentro de la mencionada familia; algo que habrá que confirmar mediante el análisis pormenorizado del genoma de otros hongos distintos a *Metarhizium*.

Las ribotoxinas fúngicas como insecticidas

La actividad insecticida de la HtA sugería que ésta podría ser la principal función de las ribotoxinas en el contexto biológico de los hongos productores. Para confirmar este supuesto, se realizó un estudio comparativo entre esta proteína y la α-sarcina, una de las ribotoxinas más representativas (resultados B1 y B2). Los resultados demuestran la casi idéntica toxicidad de ambas proteínas frente a larvas de *Galleria mellonella* y cultivos de células de insecto. Una toxicidad que es además dos órdenes de magnitud mayor que la que presentan frente a células tumorales humanas. Este resultado apoya la idea de que su función en el medio natural o es la de defender al hongo frente a insectos depredadores o la de contribuir al establecimiento de relaciones de parasitismo con ellos. En este mismo sentido, Anp también posee un nivel muy parecido de actividad tóxica frente a células de insecto. Todos estos resultados apoyan la posible utilización de estas proteínas como biopesticidas en el control de plagas, como se discute con detalle a lo largo dela Memoria (resultados B2).

Mecanismo de interacción de las ribotoxinas con el ribosoma eucariota

Las interacciones electrostáticas que establecen inicialmente las ribotoxinas con el ribosoma son una de las claves que explican su eficaz reconocimiento del SRL. No es descartable, sin embargo, que también se establezcan contactos más específicos con regiones del ribosoma que las ayuden en su especificidad. De hecho, no todas las ribotoxinas muestran la misma eficacia cuando se enfrentan a ribosomas de distintas especies, si bien, en último término todas las que se conocen sí son capaces de degradar cualquier SRL. En este contexto, ya se ha predicho la interacción entre la α-

sarcina y las proteínas ribosomales uL14 y uL6. Hay otras proteínas, de origen vegetal en este caso y con actividad glicosidasa, que comparten diana con las ribotoxinas (las *ribosome inactivating proteins* o RIP). La ricina, que también da nombre al SRL, es probablemente la más conocida. Esta ricina interacciona con proteínas ácidas del tallo para acceder al SRL. Por esto motivo, y dado el carácter tan básico de las ribotoxinas fúngicas, se pensó que sería interesante comprobar si, además de diana catalítica, estas toxinas compartían también su capacidad de interacción con el tallo. Los resultados del apartado C1 de esta Memoria indican lo contrario. La α-sarcina no utiliza este tallo ribosómico para interaccionar con el ribosoma y aproximarse al SRL. Los experimentos realizados prueban, tanto *in vivo* como *in vitro,* que el corte de este bucle de rRNA por parte de la α-sarcina se produce con la misma eficacia en presencia o en ausencia de las correspondientes proteínas ácidas en el ribosoma.

La maduración del ribosoma como nueva diana de las ribotoxinas

El proceso de ensamblaje de los ribosomas es complejo, y en él participan más de 300 factores entre proteínas y RNAs. Comienza en el nucleolo, prosigue en el nucleoplasma hasta que se forma la partícula funcionalmente activa en el citosol. Hasta ahora, la actividad de las ribotoxinas fúngicas sólo se había estudiado conribosomas maduros. Sin embargo, las partículas pre-60S también contienen rRNA susceptible de ser atacado por estas toxinas. Se ha analizado la actividad de α-sarcina sobre partículas pre-60S de levadura y el efecto del corte del SRL sobre la ruta de biogénesis del ribosoma (resultados C2). Los resultados muestran que la α-sarcina puede acceder al núcleo de las levaduras, aunque no corta el pre-rRNA 27S que contienen las partículas pre-60S en estados tempranos de maduración. La actividad ribonucleolítica de α-sarcina sí que se observó sobre partículas pre-60S citoplásmicas, que ya contienen rRNA 25S; es decir partículas en los últimos estadíos de su maduración. Por otra parte, este corte del SRL no parece provocar ningún defecto significativo en la ruta de biogénesis del ribosoma, a excepción de un aumento exacerbado de la transcripción del pre-rRNA 35S. Esto podría indicar que los intermedios de la biogénesis del ribosoma no son la principal diana de actuación de estas toxinas, sino más bien un efecto colateral de su elevada especificidad, algo que no haría sino potenciar su citotoxicidad.

Conclusiones

- La triada de lisinas del bucle 3 de α-sarcina es esencial para el reconocimiento y el corte del SRL. K111 y K114 participarían, además, en la interacción con vesículas lipídicas, y la interacción entre K114 y Y48 parece ser esencial para la catálisis.

- La deleción del bucle 2 completo, que contiene a la H82, da lugar a la pérdida de actividad catalítica de la toxina frente a todos los sustratos ensayados, pero no afecta a las interacciones con lípidos.

- Los mutantes de HtA K115E, K118E, K123E y Δ(8-15) mantienen su actividad ribonucleolítica específica, aunque ésta es menor en comparación con la proteína silvestre. La horquilla β amino terminal de la HtA y la K123 del bucle 5 parecen jugar un papel importante en la actividad insecticida de esta proteína.

- Se ha aislado y caracterizado una nueva proteína tóxica, denominada **anisoplina**, que es producida por el hongo entomopatógeno *Metarhizium anisoplae.* Presenta características moleculares y funcionales muy similares a las de HtA, lo que ha permitido clasificarla como una auténtica ribotoxina.

- Las propiedades insecticidas de la HtA no son exclusivas de ésta, sino que parecen ser una característica de todas las ribotoxinas, lo que posibilita su futura utilización en el diseño de nuevos biopesticidas para el control de plagas.

- La α-sarcina no interacciona con las proteínas ácidas del tallo ribosómico para acceder y cortar el SRL. El mecanismo de reconocimiento del SRL parece ser, por tanto, diferente al que utilizan las RIPs del tipo de la ricina.

- La α-sarcina es capaz no sólo de inactivar ribosomas maduros, sino también partículas pre-60S en las últimas etapas de la maduración. Sin embargo, el pre-rRNA 27S no es sustrato de esta ribotoxina.

Introduction

Fungi establish a complex network of biological interactions with other organisms in nature. In many cases, these involve the production of toxins. Among these toxins, ribotoxins stand out as promising candidates for their use in biotechnological applications. This family of fungal ribonucleases has been thoroughly studied since their discovery in the 1960's. The first ones to be discovered, like **α-sarcin**, were produced by *Aspergillus*, although they have also been found in other species like the entomopathogenic fungus *Hirsutella thompsonii* which produces **Hirsutellin A** (HtA).

The structure of several ribotoxins is already known at atomic resolution and mutational studies have assigned specific roles to specific residues, like their implication in catalysis, ribosome recognition or interaction with membranes. Ribotoxins are highly specific, capable of hydrolyzing a single phosphodiester bond of a rRNA conserved structure known as the sarcin-ricin loop (SRL), located in the large ribosome subunit and essential for translation. Cleavage of this specific bond inhibits protein biosynthesis and leads to cell death. Furthermore, toxicity of ribotoxins also depends on their ability to cross lipid membranes which lipid composition is important for their specificity, being more efficient on transformed or virus-infected cells. Moreover, there must be specific interactions with ribosome regions that lead them to the SRL, some of them have already been previously proposed.

Although the native function of ribotoxins in nature has not been assigned yet, some studies have proven their insecticidal properties, supporting their role in parasitism and/or defense mechanisms against predators. Within the same idea, the detailed study of the structure and function of this family of toxic proteins has pointed out their potential as biotechnological weapons. Much has been done regarding their use as antitumoral immunotoxins, and their implication in allergies and *Aspergillus*-related infections. In this Thesis, we propose the use of fungal ribotoxins as biopesticides, and also as tools for the study of ribosome biogenesis which in the long term may be useful to understand ribosome-related diseases, the so-called ribosomopathies.

Objectives

- Study of structure-function relationships in ribotoxins with specific focus on the role of α-sarcin loops 2 and 3 residues, as well as the N-terminal β hairpin and loop 5 residues of HtA.

- Characterization of a new ribotoxin, anisoplin, similar to HtA and produced by the fungus *Metarhizium anisopliae*.

- Comparative study of the insecticidal properties of the most representative fungal ribotoxins, evaluating their potential use as biological control agents as an alternative to classical pesticides.

-Study of the interaction of fungal ribotoxins with the eukaryotic ribosome using the yeast *Saccharomyces cerevisiae* as the model organism, analyzing whether the ribosomal stalk is involved in the mechanism of interaction of α-sarcin with the ribosome.

-Determination of the effect of fungal ribotoxins on the eukaryotic ribosome biogenesis pathway.

Results and discussion

Structure-function relationships of fungal ribotoxins

We first studied specific aspects of the structure and function of ribotoxins. Mainly, the role of loops 2 and 3 of α-sarcin (results A1) and loop 5 and the N-terminal β hairpin of HtA (results A2).

The mutants of loop 3 of α-sarcin herein studied showed that this region is crucial for its specific catalytic activity. K111 and K114 are involved in approaching lipid vesicles, contributing to the vesicle aggregation processes observed in the presence of this protein. Moreover, we have described the existence of a network of interactions between K114 and Y48, a key residue in the specificity of the ribotoxin. Regarding loop 2, H82 does not seem to be essential but its substitution for glutamine (H82Q) enhances the lipid vesicles permeabilizing ability.

Substitution of residues 79 to 93 of loop 2 in α-sarcin for the corresponding stretch of HtA (ADAI) considerably shortens it, affecting the geometrical arrangement of the active site and causing the loss of its enzymatic activity. This deletion, however, does not change its lipid interacting abilities, implying that this sequence is not involved in the interaction with membranes.

The N-terminal β hairpin of HtA is considerably shorter and contains less positive charges than α-sarcin. To check the hypothesis that this shorter hairpin might be compensated by the greater length and different orientation of loop 5, we produced different mutant variants of HtA. The deletion of the N-terminal β hairpin resulted in a protein that still retained the specific ribonucleolytic activity against ribosomes and the SRL, but this activity was lower than that one observed for wild type HtA. The same happened with the other single-residue mutations of loop 5 studied. These results suggest that variations of the microenvironment of the active sites differentially affect both ribotoxins, being HtA more adaptable and able to compensate changes without losing its activity. The active site of α-sarcin would be

then more rigid, explaining why modification of its catalytic residues renders completely inactive mutants.

Finally, analysis of the genome of the entomopathogenic fungus *Metarhizium anisopliae* revealed the existence of a protein that shares 70% of sequence identity with HtA. This protein was indeed a ribotoxin, named **anisoplin** (Anp) (results A3). Anp was produced as a recombinant protein in *Escherichia coli* and structurally and functionally characterized, showing the specific ribonucleolytic activity characteristic of ribotoxins. So far, the smaller size of HtA made this toxin an exception among ribotoxins. The discovery of Anp as a true ribotoxin suggests the possibility that there is a new subgroup of proteins within ribotoxins. This hypothesis should be further studied by analyzing in detail the genome of other fungi apart from *Metarhizium*.

Fungal ribotoxins as insecticides

The insecticidal activity of HtA suggested that this could be the main function of ribotoxins in the biological context of the fungus. To prove this assumption, we performed a comparative study with this protein and α-sarcin, one of the most representative ribotoxins (results B1 and B2). Results show that both proteins share almost identical toxicity against *Galleria mellonella* larvae and insect cell cultures. This toxicity is two orders of magnitude higher than the one they display against human tumor cells, supporting the idea that in the natural environment these ribotoxins either defend the fungus against predator insects or enable the establishment of parasitic relationships with them. In this regard, Anp also shares this toxicity against insect cells. This study would support the potential use of these proteins as biopesticides in biological control, as it is discussed along this Thesis (results B2).

Mechanism of interaction between ribotoxins and the eukaryotic ribosome

Electrostatic interactions initially established between ribotoxins and the ribosome are important to explain their efficient recognition of the SRL. It cannot be discarded, however, that more specific contacts with other ribosomal regions are established to enhance their specificity. In fact, not all ribotoxins show the same efficiency when assayed against ribosomes of different origins, whereas they can all degrade the SRL, regardless its origin. In this direction, it has already been predicted the interaction between α-sarcin and ribosomal proteins uL14 and uL6. Other toxic proteins such as ricin (the so-called *ribosome inactivating proteins*, RIP), of plant origin and glycosidase activity, share target with ribotoxins. It has been shown that ricin interacts with the acidic proteins of the ribosomal stalk to access the SRL. This fact, together with the basic character of fungal ribotoxins, led us to check if apart from sharing the SRL as target, these toxins also shared the ability to interact with the ribosomal stalk. The results herein presented discard this hypothesis (results C1). α-sarcin does not use the ribosomal stalk to interact with the ribosome and approach the

SRL. Both *in vivo* and *in vitro* experiments prove that the cleavage of the SRL by α-sarcin is equally efficient in the presence or absence of acidic proteins in the ribosome.

Ribosome maturation as a new target of ribotoxins

Assembly of ribosomes is a complex process in which more than 300 factors, both proteins and RNAs, are involved. It begins in the nucleolus, continues in the nucleoplasm until the functional particle is formed in the cytoplasm. Until now, the activity of fungal ribotoxins has only been studied with mature ribosomes. However, pre-60S particles also contain rRNA susceptible of being attacked by these toxins. For that reason, the activity of α-sarcin on these pre-60S particles has been analyzed in this Thesis, as well as the cleavage of SRL along the route of ribosome biogenesis (results C2). Results show that, even though α-sarcin can enter yeast nucleus, it is not able to cleave the 27S pre-rRNA contained in early pre-60S particles. The ribonucleolytic activity of α-sarcin was only observed on late pre-60S particles, mainly cytoplasmic, which contain mature 25S rRNA. Besides, SRL cleavage does not considerably affect ribosome biogenesis apart from an enhanced transcription of the 35S pre-rRNA. This could indicate that intermediate events of ribosome biogenesis are not the main target of fungal ribotoxins but a collateral effect of their high specificity, enhancing their toxicity.

Conclusions

- The triad of lysines in loop 3 of α-sarcin is essential for SRL recognition and cleavage. K111 and K114 would indeed participate in the interaction with lipid vesicles, and the interaction between K114 and Y48 seems to be essential for catalysis.

- Deletion of loop 2 of α-sarcin, which contains H82, leads to the loss of catalytic activity of the toxin against the substrates used, but does not affect the interactions with lipids.

- The mutant variants of HtA K115E, K118E, K123E and Δ(8-15) retain their specific ribonucleolytic activity, although it is lower than in the wild type protein. The N-terminal β hairpin of HtA and K123 in loop 5 seem to play an important role in the insecticidal activity of this protein.

- A new toxic protein, named **anisoplin** (Anp), has been characterized. It is produced by the entomopathogen fugus *Metarhizium anisopliae*, and has similar molecular and functional features than HtA. It can be considered a ribotoxin.

- The insecticidal properties of HtA seem to be characteristic of all ribotoxins, allowing the future use of these proteins in the design of new biopesticides.

- α-Sarcin does not interact with the acidic proteins of the ribosomal stalk to access and cleave the SRL. The mechanism of recognition of the SRL is therefore quite different than the one used by plant RIPs like ricin.

- α-Sarcin is capable of inactivating not only mature ribosomes, but also late pre-60S particules. However, 27S pre-rRNA is not cleaved by this ribotoxin.

Introducción/Introduction

Las ribotoxinas fúngicas como herramientas biotecnológicas: un conjunto de aplicaciones multidisciplinares.

Resumen

En la naturaleza, los hongos establecen una compleja red de interacciones biológicas con otros organismos. En muchas ocasiones, éstas conllevan la producción de toxinas de todo tipo. Algunas de estas toxinas presentan una elevada actividad insecticida, lo que ha llevado a pensar que puedan participar en la defensa del hongo frente a diferentes depredadores. De entre todas estas toxinas destacan, debido a su potencial uso en distintas aplicaciones biotecnológicas, las ribotoxinas. Esta familia de proteínas está constituida por ribonucleasas (RNasas) altamente específicas, cuya diana es una secuencia de RNA ribosomal conservada universalmente, el lazo sarcina/ricina. Su potencial biotecnológico ya ha sido demostrado, pues se han utilizado con éxito como agentes antitumorales, formando parte de inmunotoxinas. A continuación se discute la posible función insecticida de estas proteínas, así como su potencial uso como herramientas biotecnológicas en distintas aplicaciones, desde su utilización en terapias frente al cáncer o la alergia, hasta su empleo como bioinsecticidas en el control de plagas o incluso como herramientas para el estudio de algunas ribosomopatías humanas.

Introducción

Casi cualquier nicho ecológico del planeta está poblado por organismos que establecen entre ellos una gran variedad de interacciones biológicas, desde el mutualismo al antagonismo, enfocadas sobre todo a la búsqueda de recursos para su supervivencia (luz, nutrientes, agua, etc.). En particular, los hongos constituyen una rica fuente de nitrógeno y fósforo para artrópodos, y por ello son atacados constantemente por fungívoros como colémbolos, ácaros e insectos (Berenbaum y Eisner 2008). Sin embargo, también pueden establecer relaciones de mutualismo, como las que presentan con las hormigas arrieras con el hongo que usan como alimento. De este modo, aparece en la naturaleza una compleja red de interacciones que incluye la depredación, defensa y alimentación. En este contexto, los hongos producen una amplia variedad de toxinas, muchas de ellas proteicas. Estas toxinas pueden ser secretadas por muy diferentes motivos, aunque la autodefensa y el correcto desarrollo del hongo parecen ser sus principales funciones.

Las ribotoxinas fúngicas constituyen una familia de RNasas extracelulares que se han estudiado con detalle desde su descubrimiento a principios de los años 60. No son las únicas RNasas producidas por hongos, pero sí las únicas que presentan actividad citotóxica. Estas proteínas son producidas por distintas especies de hongos, generalmente del género *Aspergillus*, aunque también las producen otros hongos

entomopatógenos como *Hirsutella thompsonii* (Martínez-Ruiz et al. 1999, Martínez-Ruiz et al. 1999, Lacadena et al. 2007, Herrero-Galán et al. 2008). Son extremadamente específicas, ejerciendo su actividad ribonucleolítica sobre la molécula mayor del rRNA. Desde este punto de vista podrían incluirse en el grupo de proteínas inactivantes del ribosoma (*ribosome-inactivating proteins*, RIPs), aunque este término suele referirse a las toxinas con actividad N-glicosidasa y de origen vegetal, entre las que destaca la ricina, una toxina que se encuentra en las semillas de la planta *Ricinus communis*. Tanto las RIPs como las ribotoxinas tienen como diana la misma secuencia universalmente conservada de rRNA, conocida como lazo sarcina/ricina (sarcin/ricin loop, SRL), bien despurinando un único nucleótido o bien cortando un único enlace fosfodiester, respectivamente. No obstante, los mecanismos por los cuales ambos tipos de toxina reconocen esta diana parecen ser bastante diferentes (Chiou et al. 2008, Olombrada et al. 2014).

A pesar de que la función biológica de las ribotoxinas no se ha esclarecido aún, muchos estudios han demostrado las propiedades insecticidas de al menos dos de estas proteínas, apoyando su participación en mecanismos de defensa y parasitismo (Brandhorst et al. 1996, Brandhorst et al. 2001, Olombrada et al. 2013). El estudio detallado tanto de la estructura como de la función de esta familia de proteínas tóxicas ha demostrado su potencial uso como herramientas biológicas, bien como bioinsecticidas en el control de plagas (Olombrada et al. 2014), bien como agentes antitumorales frente a algunos tipos de cáncer (Tome-Amat et al. 2015c). Además, se podrían utilizar también como herramientas específicas en el estudio de enfermedades humanas relacionadas con el ribosoma, las llamadas ribosomopatías (Narla y Ebert 2010, Nakhoul et al. 2014).

Las ribotoxinas fúngicas

Las ribotoxinas no son las únicas RNasas producidas por hongos. De hecho, forman parte de un grupo más amplio de RNasas fúngicas extracelulares en el que las ribotoxinas son las únicas que presentan actividad citotóxica. Estas ribotoxinas son de mayor tamaño que las RNasas fúngicas no tóxicas, pero siguen siendo pequeñas proteínas de unos 150 aminoácidos y, generalmente, básicas. La principal diferencia estructural entre las RNasas no tóxicas, como las RNasas T1 o U2, y las ribotoxinas sería que estas últimas poseen bucles más largos, cargados positivamente, lo que sugiere que dichos bucles son la base estructural de su citotoxicidad (Martínez-del-Pozo et al. 1988). La identificación de las características estructurales que hacen a las ribotoxinas tan eficientes en su acción citotóxica ha permitido dar un gran paso hacia su utilización como herramientas específicas contra plagas de insecto o en distintas patologías humanas. Hasta el momento, las ribotoxinas mejor caracterizadas son la α-sarcina, la restrictocina, la Aspf1 y la hirsutelina A (HtA). Las tres primeras presentan un elevado grado de conservación, con identidades de secuencia por encima del 85%.

Sin embargo, HtA, una ribotoxina producida por *H. thompsonii*, solo comparte un 25% de identidad de secuencia con ellas (Figura 1). Esta observación confirma que la presencia de ribotoxinas entre los hongos está más extendida de lo que inicialmente se pensaba, y no se restringe solo al género *Aspergillus* (Liu et al. 1995, Martínez-Ruiz et al. 1999, Martínez-Ruiz et al. 1999, Varga y Samson 2008).

La actividad ribonucleolítica de las ribotoxinas es extremadamente específica, dirigida a una secuencia de rRNA muy conservada en cualquier ribosoma conocido, el SRL (Figura 1). Esta secuencia se localiza en la molécula grande de rRNA de la subunidad mayor del ribosoma. Durante la traducción, el SRL junto con la región de unión a uL11, el tallo L7/L12 (P1/P2 en levadura) y las proteínas uL6 y uL14, constituye un sitio de unión a factores de elongación que es esencial para el correcto funcionamiento del ribosoma (Garcia-Ortega et al. 2010, Voorhees et al. 2010). El SRL es además la secuencia más larga de rRNA conservada universalmente, y su estructura es muy similar en ribosomas de arqueas, bacterias y eucariotas. Consiste en una horquilla distorsionada cuyas características estructurales más significativas son un tetrabucle GAGA y un motivo de G prominente. Las ribotoxinas cortan un único enlace fosfodiester del tetrabucle GAGA, produciendo un pequeño fragmento de 300-400 nucleótidos de rRNA, conocido como fragmento α. Esta observación avala su extraordinaria especificidad dado que un ribosoma contiene más de 5000 enlaces de este tipo. Estas toxinas son RNasas ciclantes que presentan un mecanismo ácido-base de ruptura endonucleolítica de RNA en dos etapas, como los otros miembros de la familia de la RNasa T1 (Lacadena et al. 1998, Lacadena et al. 2007). El corte de este enlace fosfodiester inhibe la biosíntesis de proteínas y produce la muerte celular por apoptosis (Olmo et al. 2001).

La estructura tridimensional de varias ribotoxinas se conoce con resolución atómica (Figura 1) (Yang y Moffat 1996, Pérez-Cañadillas et al. 2000, Viegas et al. 2009). El análisis mutacional ha permitido la asignación de papeles concretos a diferentes residuos (implicación en la catálisis, reconocimiento del SRL y del ribosoma, interacción con la membrana, etc.). En el caso de la α-sarcina, los residuos catalíticos son His 50, Glu 96 e His 137. Glu 96 actuaría como base general y la His 137 como ácido general (Lacadena et al. 1995, Martinez-Ruiz et al. 1999). La His 50 contribuiría a la estabilización del estado de transición. Estos tres residuos son esenciales para la inactivación específica de los ribosomas. Otros estudios similares han mostrado cómo Tyr 48, Arg 121 y Leu 145, aunque no son esenciales, contribuyen a la actividad catalítica de la α-sarcina. Los residuos catalíticos equivalentes en HtA (His 42, Glu 66 e His 113) fueron identificados por comparación de su estructura con la de otras ribotoxinas. Dicha comparación mostró cómo efectivamente los residuos esenciales están conservados, si bien la HtA también presenta algunas características más parecidas a las de las RNasas no tóxicas del tipo T1 (Phe 126 en lugar de la equivalente Leu 145, por ejemplo) o, incluso, otras características completamente nuevas (Asp 40

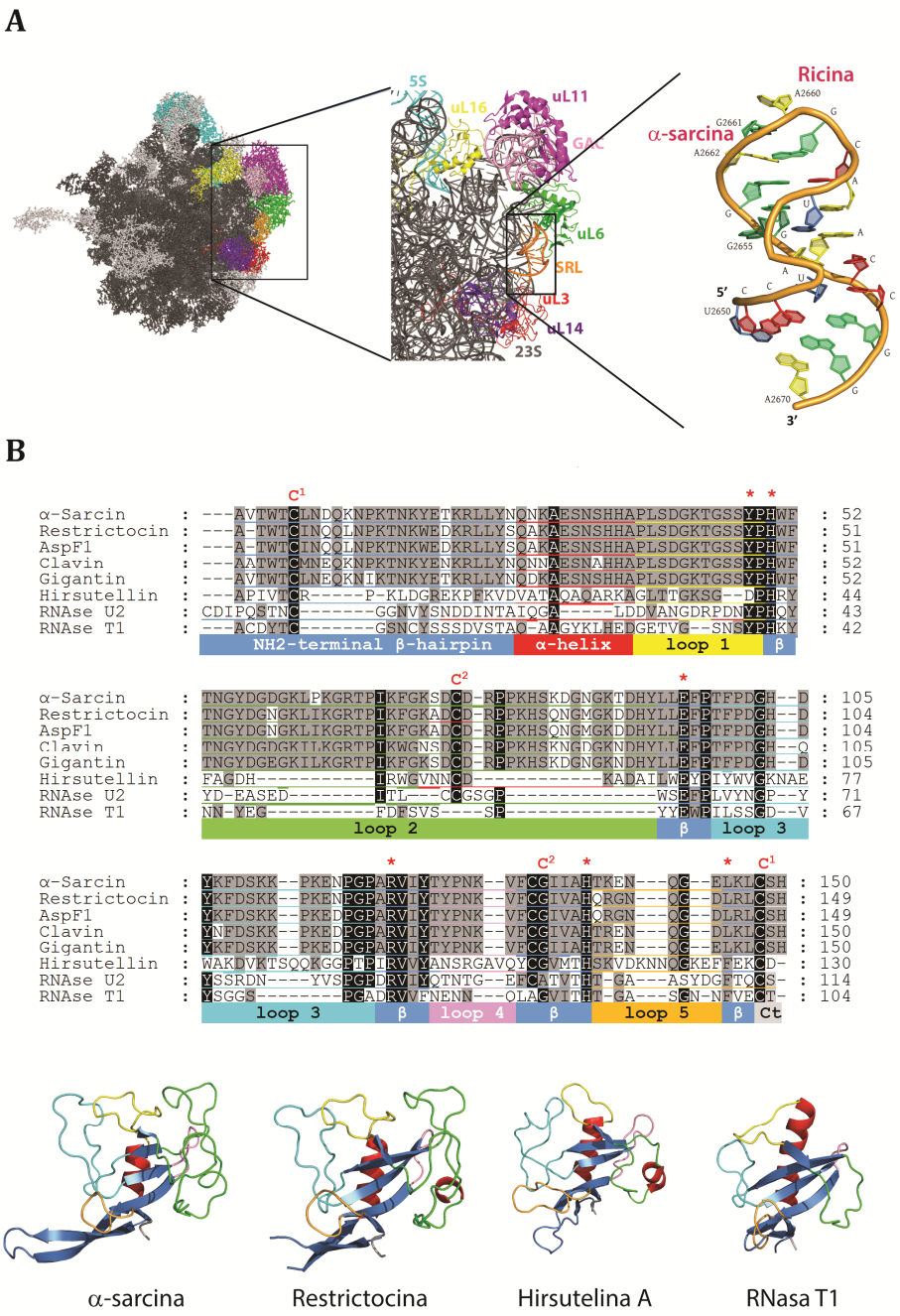

Figura 1. A) Estructura tridimensional de la subunidad mayor del ribosoma de *Escherichia coli* (PDB ID 2AW4). Aparecen destacadas en color las proteínas próximas al SRL: uL3 (rojo), uL6 (verde), uL11 (rosa), uL14 (morado) y uL16 (amarillo). También se muestran los rRNA 23S (gris) y 5S (cian). GAC, centro de activación de GTPasa. La ampliación destaca la zona cercana al SRL, y el propio SRL, donde se indica el enlace cortado por la α-sarcina y la adenina que despurinan otras RIPs como la ricina. B) Alineamiento de secuencia de las RNasas fúngicas más representativas. Se indican los residuos conservados del centro catalítico (*) así como las cisteínas, también conservadas, que forman los puentes disulfuro (C). Los aminoácidos invariables aparecen destacados en negro, y los elementos de estructura secundaria se indican en distintos colores en la parte inferior del alineamiento. También se muestran las estructuras tridimensionales de la RNasa T1 y de las ribotoxinas más representativas (PDB IDs: 1DE3, 1AQZ, 2KAA, 9RNT). Estos diagramas se generaron usando el programa PyMOL.

en lugar de Tyr 48) (Herrero-Galán et al. 2012). También mediante mutagénesis se demostró que el centro activo de la HtA podría poseer un microambiente más adaptable que el de la α-sarcina, ya que ninguno de los residuos catalíticos identificados parece ser indispensable para la catálisis, como sí sucede con ésta (Herrero-Galán et al. 2012).

La toxicidad de las ribotoxinas resulta de la combinación de su actividad ribonucleolítica altamente específica y de su habilidad para cruzar membranas lipídicas. Hasta el momento no se han descrito receptores proteicos que expliquen esta capacidad para internarse a través de las membranas. Por ello, aunque las ribotoxinas son capaces de inactivar cualquier ribosoma, la composición lipídica de la membrana celular es crítica a la hora de determinar su especificidad citotóxica. Se ha descrito, por ejemplo, cómo las ribotoxinas son más eficientes en células transformadas o infectadas por virus, muy probablemente debido a una permeabilidad y/o composición de la membrana celular alteradas (Turnay et al. 1993, Olmo et al. 2001). En este sentido es importante destacar que las membranas de las células de insecto tienen una composición lipídica particular, siendo más finas y fluidas, lo que parece hacerlas más sensibles y accesibles a la actividad citotóxica de las ribotoxinas (Marheineke et al. 1998, Olombrada et al. 2013). El uso de vesículas lipídicas modelo ha permitido demostrar que la α-sarcina interacciona con vesículas ricas en fosfolípidos ácidos. Esta interacción produce la agregación de estas vesículas, seguida de su fusión, con intercambio de fosfolípidos, que finaliza con la liberación de su contenido acuoso (Figura 2) (Gasset et al. 1989, Gasset et al. 1990). No obstante, éste no parece ser un mecanismo general para todas las ribotoxinas, ya que aunque la HtA no promueve agregación de vesículas, sí que parece poseer mayor capacidad de permeabilización de membranas, al menos en los experimentos de liberación de contenidos acuosos (Herrero-Galán et al. 2008, Olombrada et al. 2013). En la α-sarcina parece que la región que comprende los residuos 116-139 participa en la interacción hidrofóbica con las membranas (Mancheño et al. 1995, Mancheño et al. 1998), y recientemente se ha mostrado que las lisinas 111 y 114 también participarían en las interacciones electrostáticas que permiten el contacto entre vesículas necesario para su fusión (Castaño-Rodríguez et al. 2015). En cuanto a la HtA, parece que serían sus triptófanos 71 y 78 los que participarían en la permeabilización de las membranas (Herrero-Galán et al. 2012). Esta habilidad para interaccionar con membranas lipídicas también se ha asociado con la horquilla β amino-terminal de las ribotoxinas. La deleción de esta región cargada positivamente produce una RNasa activa, pero no tóxica, con alteraciones precisamente en esta capacidad (García-Ortega et al. 2001). Es precisamente en esta región donde las ribotoxinas muestran también mayor variabilidad. En la HtA, además, la horquilla β amino-terminal es mucho más corta (Figura 1), una alteración estructural que podría ser compensada por la mayor extensión del bucle 5, que también posee una mayor cantidad de cargas positivas (Herrero-Galán et al. 2012).

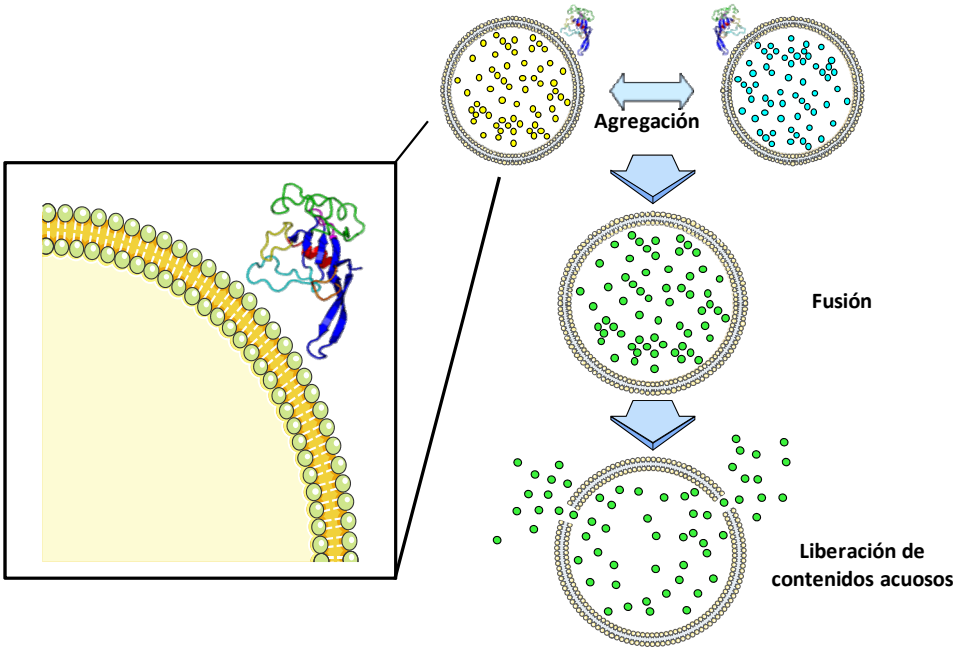

Figura 2. Esquema del mecanismo de fusión lipídica inducido por la α-sarcina. Esta fusión acaba produciendo vesículas inestables, lo que se refleja en la liberación de su contenido acuoso.

La superficie de las ribotoxinas está cargada positivamente, lo que podría favorecer el establecimiento de interacciones electrostáticas no sólo con las membranas, sino también con el ribosoma (García-Mayoral et al. 2005, Korennykh et al. 2006, Álvarez-García et al. 2009). Una idea que encaja con la observación de que la elevada especificidad de las ribotoxinas requiere la participación de elementos adicionales, más allá de los que conforman el centro activo, para poder interaccionar con otras regiones del ribosoma y reconocer el SRL. En este sentido, se ha propuesto que una región rica en lisinas del bucle 3 interaccionaría con las cargas negativas de los fosfatos en el entorno de la G prominente del SRL, mientras que los residuos 51 a 55 del bucle 2 de la α-sarcina, y algunos de sus residuos del bucle 5, serían los encargados de contactar con el tetrabucle GAGA, que después es cortado por la toxina (Yang et al. 2001, García-Mayoral et al. 2005). El modelado del reconocimiento del ribosoma por las ribotoxinas, utilizando las estructuras del ribosoma de *Haloarcula marismortui* y de la α-sarcina, sugiere también que, además de estas posibles interacciones con el SRL, la α-sarcina podría interaccionar con algunas proteínas del ribosoma cercanas a dicho lazo de rRNA. Este modelado predice que una secuencia corta del bucle 2 contactaría con la proteína uL6, mientras que los residuos 11 a 16 de la horquilla β amino-terminal interaccionarían con la proteína uL14 (Figura 1). Esta segunda secuencia muestra además gran similitud con una región del factor de elongación 2 (EF-2) de *Saccharomyces cerevisiae* (García-Mayoral et al. 2005), que se une a la misma zona del ribosoma para ejercer su función. Basándose en estas observaciones, parece razonable pensar que otras regiones del ribosoma encargadas de interaccionar con factores de elongación puedan también establecer interacciones con estas toxinas. Por ejemplo,

las proteínas del tallo ribosómico. En este sentido, ha sido demostrado que algunas RIPs, como la ricina, la tricosantina y algunas toxinas del tipo Shiga, sí que lo hacen (Chiou et al. 2008, Tumer y Li 2012). De hecho, esta estructura protuberante del ribosoma sirve a algunas de estas RIPs como plataforma de anclaje para su posterior reconocimiento del SRL. Se trata sin embargo de una situación compleja ya que, a pesar de compartir la misma diana, no todas las RIPs se comportan de la misma manera. Por ejemplo, la proteína antiviral del carmín (*pokeweed antiviral protein*, PAP), que también es una RIP de plantas, no establece ningún tipo de interacción con las proteínas del mencionado tallo ribosómico (Ayub et al. 2008). Tampoco parece ser éste el caso de las ribotoxinas, al menos en el caso de α-sarcina (Olombrada et al. 2014). Un resultado que sugiere que el mecanismo por el cual las ribotoxinas reconocen y se aproximan al ribosoma es muy diferente del que utilizan RIPs como la ricina.

El género *Aspergillus* y otros hongos productores de ribotoxinas.

Las ribotoxinas fúngicas fueron descubiertas en los años 60 durante un programa de búsqueda de antibióticos y agentes antitumorales en el Departamento de Salud de Michigan. El hongo *Aspergillus giganteus* producía una proteína capaz de inhibir el crecimiento de sarcomas y carcinomas inducidos en ratones (Olson y Goerner 1965), a la que se llamó α-sarcina. Poco después, otras dos proteínas con actividad similar, restrictocina y mitogilina, fueron descubiertas en *Aspergillus restrictus*. Más recientemente se ha publicado que todas las especies asignadas al género *Aspergillus* sección *Clavati* contienen genes para ribotoxinas (Varga y Samson 2008). Este género *Aspergillus* comprende un grupo complejo y ubicuo de hongos filamentosos formado por más de 185 especies, incluyendo patógenos humanos, como *Aspergillus fumigatus*, así como otras especies de interés en la industria para la producción de comida o enzimas, como *Aspergillus oryzae* (Machida et al. 2005). Curiosamente, *A. fumigatus*, un patógeno oportunista humano, produce la ribotoxina Aspf1 (Figura 1). El genoma de estas dos especies, junto con el de *Aspergillus nidulans*, ya se conoce desde hace algunos años (Galagan et al. 2005). Es más, en 2008 ya se había completado la secuencia de más de siete especies de *Aspergillus*, lo cual constituye un prometedor avance en el estudio de la biología de los hongos y su evolución. De hecho, la secuenciación del genoma de otros hongos podría utilizarse para la identificación de nuevas especies productoras de ribotoxinas. Además, la comparación de estos genomas con los de otras especies no productoras podría ayudar a elucidar la función biológica de estas proteínas.

La mayoría de hongos filamentosos posee un ciclo de vida complejo. Como otros *Ascomycota*, algunos *Aspergilli* pueden alternar ciclos de reproducción sexual y asexual (Figura 3). *A. nidulans* es un buen ejemplo para describirlos, ya que posee ambos tipos de reproducción (Casselton y Zolan 2002). En la reproducción sexual, se

suceden la cariogamia (fusión nuclear) y meiosis. El hongo desarrolla un cuerpo fructífero, el cleistotecio, que produce esporas sexuales o ascosporas. Hasta el momento, sólo se ha observado este tipo de reproducción en algunos *Aspergilli*. Durante el ciclo asexual, el micelio, que se desarrolla a partir de una espora haploide, se diferencia en muchas esporas idénticas, conidiosporas, capaces de formar una nueva red de hifas. No existen muchos estudios acerca de la expresión de ribotoxinas en su entorno natural, ni su papel en el ciclo vital del hongo. La presencia de ribotoxinas se ha detectado, sin embargo, durante la reproducción asexual de *A. restrictus*. Este hongo produce la ribotoxina restrictocina, que es prácticamente indistinguible de α-sarcina, en un momento muy específico del desarrollo del hongo: las primeras etapas de la formación del conidióforo (Brandhorst y Kenealy 1992). Los primeros estudios que revelaron este dato se realizaron en medio de cultivo líquido, donde el hongo crecía sumergido. En ellos se observaba una correlación entre la aparición de restrictocina en dicho medio y algunas estructuras diferenciadas del hongo (Yang y Kenealy 1992). Aún así, estas estructuras no se desarrollan adecuadamente en un entorno líquido. Por ello, estudios posteriores utilizando medio de cultivo sólido, que se asemeja más al ambiente natural de estrés hídrico en el que se encuentra *A. restrictus*, revelaron que los niveles de restrictocina aumentaban progresivamente hasta el momento de la maduración de los conidios, siendo degradada justo después (Brandhorst y Kenealy 1992). En medio sólido, la restrictocina aparecía exclusivamente en la superficie de las fiálides y desaparecía tras la maduración de los conidios (Figura 3). Posibles explicaciones para este patrón de expresión de las ribotoxinas incluyen la posibilidad de que la restrictocina participe en el proceso de maduración de los conidios, o que simplemente tenga un papel protector durante dicha maduración frente a depredadores, hasta que el desarrollo se haya completado. Una vez terminada la maduración, la restrictocina sería degradada para que los insectos que se alimentan del hongo puedan transportar y diseminar las esporas a otros lugares. En línea con la hipótesis de una función protectora de las ribotoxinas durante la maduración del conidio, se han realizado algunos estudios sobre el comportamiento alimenticio del escarabajo fungívoro *Carpophilus freemani* durante la producción de restrictocina (Brandhorst et al. 1996). Los individuos adultos de *C. freemani* se alimentaban menos de *A. restrictus* durante la formación de los conidios, algo que no ocurría cuando se alimentaban de *A. nidulans*. Esta alteración en la alimentación de los insectos se puede relacionar con un incremento de la producción de restrictocina en *A. restrictus* mientras que *A. nidulans* no produce ninguna ribotoxina. Además, la expresión heteróloga de restrictocina en *A. nidulans* también desfavorecía la alimentación de *C. freemani* adultos (Brandhorst et al. 2001), relacionando este efecto con la producción de la ribotoxina.

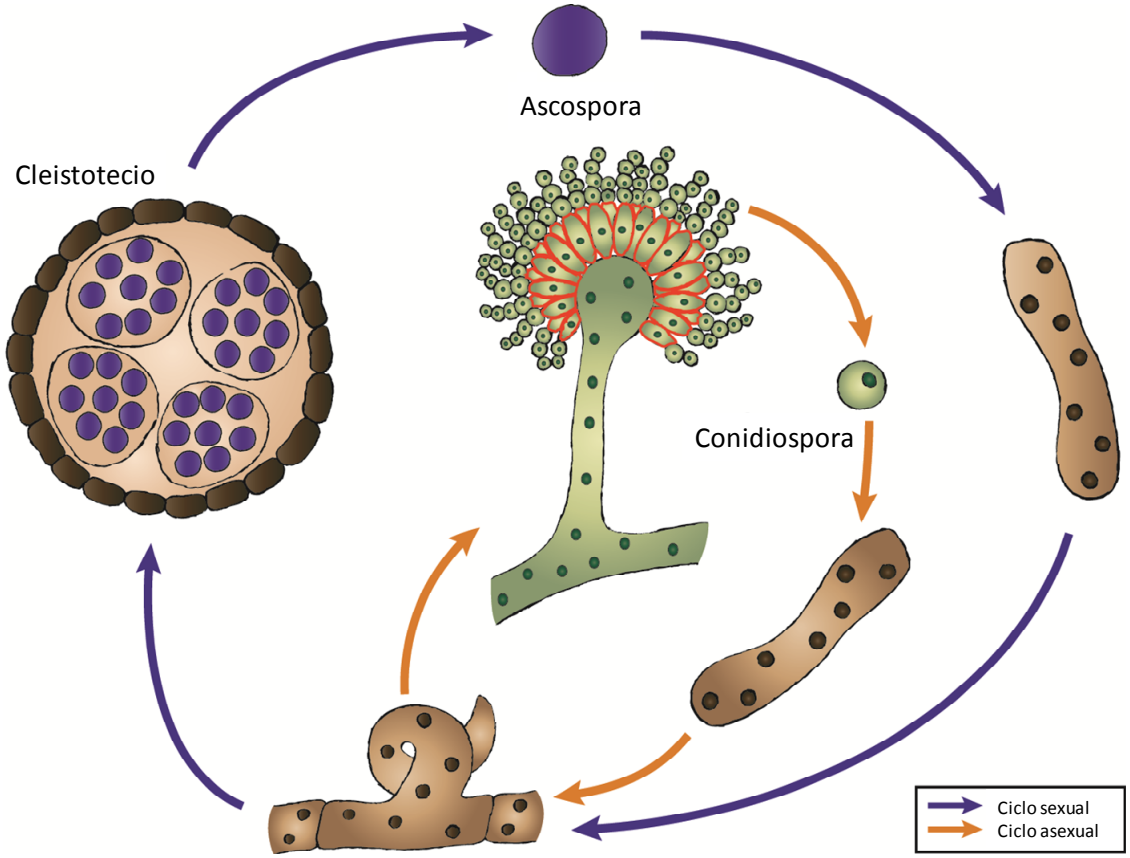

Figura 3. Reproducción de *Aspergillus* y posible localización de las ribotoxinas. *Aspergillus* puede poseer un ciclo reproductivo sexual o asexual. Durante el ciclo sexual, el micelio forma un cuerpo fructífero, el cleistotecio, que contiene las ascosporas que se liberan al medio ambiente para formar nuevas hifas. En la reproducción asexual, el micelio se diferencia en esporas asexuales idénticas, las conidiosporas. Las ribotoxinas se producirían durante la maduración de los conidios, localizándose en los extremos de las fiálides (rojo). Además de estos ciclos, *Aspergillus* también puede reproducirse de manera parasexual (no mostrado en la figura).

Como se ha comentado anteriormente, la producción de ribotoxinas no se restringe sólo al género *Aspergillus* (Liu et al. 1995, Liu et al. 1996, Martínez-Ruiz et al. 1999, Martínez-Ruiz et al. 1999, Varga y Samson 2008). De hecho, *Hirsutella* incluye más de 50 especies que infectan a una gran variedad de invertebrados y. al menos uno de ellos, *H. thompsonii,* también produce una ribotoxina. Este hongo es el causante de epidemias naturales en poblaciones de ácaros eriofioides. En condiciones *in vivo*, sus conidios se adhieren a la cutícula del hospedador, germinan y penetran a través de ella. Las hifas crecen en el interior del artrópodo y se reproducen de manera asexual, contactando con nuevos hospedadores para empezar un nuevo ciclo de infección (Peng et al. 2002). En los años 90 se realizaron estudios sobre la toxicidad del filtrado del medio de cultivo de este hongo, mostrando una alta especificidad por la subclase *Acari*, aunque también resultaba tóxico para otros artrópodos como polillas, moscas y larvas de mosquito (Vey et al. 1993). Es más, el papel de las toxinas en la patogénesis de la infección por *H. thompsonii* también se ha investigado. Inicialmente se observó

que otros hongos entomopatógenos, como *Metarhizium anisopliae* o *Bauveria bassiana,* producían péptidos cíclicos que resultaban ser tóxicos para insectos (de Bekker et al. 2013). Estos péptidos inducen parálisis inmediata y mortalidad temprana cuando se inyectan en larvas de la polilla *Galleria mellonella*, por ejemplo. Análogamente, si se inyectaba el filtrado del medio de cultivo de *H. thompsonii* se observaban síntomas similares, si bien más lentos, pero igual de letales para las larvas de la polilla. Fueron estas investigaciones las que llevaron a la identificación de la HtA como una proteína producida por *H. thompsonii var. thompsonii* con actividad insecticida frente a larvas de *G. mellonella* y *Aedes aegypti* (Liu et al. 1995, Mazet y Vey 1995). Esta toxina se purificó y caracterizó, observándose que era capaz de inactivar ribosomas (Liu et al. 1996) según un mecanismo idéntico al de las ribotoxinas. El análisis de 162 cepas reveló la presencia del gen de HtA en 100 de ellas, pero la expresión de HtA tenía muy baja correlación con la mortalidad inducida en *G. mellonella* por los filtrados del hongo, concluyéndose que debía haber factores de toxicidad adicionales involucrados en la actividad insecticida del hongo (Maimala et al. 2002). No existen todavía estudios sobre la localización exacta de HtA en el hongo, como los llevados a cabo con *A. restrictus*. Por ello, aún no se puede relacionar la producción de esta toxina con ningún estadío de su desarrollo. Poco después de su descubrimiento, HtA se relacionó ya directamente con las ribotoxinas, dado que era capaz de cortar específicamente rRNA de células Sf9 de *Spodoptera frugiperda*, inhibiendo el crecimiento celular (Liu et al. 1995). Se sometió a HtA a una caracterización estructural y funcional exhaustiva, demostrando que, a pesar de su menor tamaño y poca identidad de secuencia, se trataba de una auténtica ribotoxina (Herrero-Galán et al. 2008, Viegas et al. 2009, Herrero-Galán et al. 2012, Herrero-Galán et al. 2012).

De hecho, fue esta caracterización la que permitió plantear la posibilidad de que las ribotoxinas estuvieran actuando como insecticidas naturales. Esta idea está también apoyada por el hecho de que *Aspergillus*, hasta ahora el mayor productor de ribotoxinas, comparte nicho ecológico con varios insectos, y como método de supervivencia podría haber desarrollado la producción de armas defensivas como las ribotoxinas. Además, la inclusión de restrictocina pura en la dieta de los insectos es capaz de matar larvas de *C. freemanii* y *S. frugiperda*, y los individuos adultos de *C. freemani* dejan de alimentarse, cuando el hongo produce esta ribotoxina, como ya se ha comentado (Brandhorst et al. 1996). La comparación directa de la actividad de HtA y α-sarcina demostró que ésta última es casi tan tóxica como HtA frente a larvas, cultivos celulares y ribosomas de insecto (Olombrada et al. 2013), y la caracterización bioquímica y enzimática frente a células de insecto puso de manifiesto que ambas inhiben la síntesis de proteínas y causan la liberación del fragmento α característico del corte del SRL (Olombrada et al. 2013), como ya se había demostrado con ribosomas de otros organismos (Lacadena et al. 2007, Herrero-Galán et al. 2008, Garcia-Ortega et al. 2010). En las condiciones ensayadas, sin embargo, HtA es

significativamente más activa que α-sarcina frente a ribosomas aislados, sugiriendo que el paso a través de las membranas celulares puede ser limitante para su actividad citotóxica. Por supuesto, en su ambiente natural su eficiencia puede estar influida por otros factores adicionales, como su tasa de producción o de secreción, o su estabilidad en el ambiente en el que se produce.

El hecho de que las ribotoxinas puedan inactivar cualquier ribosoma conocido hasta la fecha plantea la pregunta de cómo los hongos protegen a sus propios ribosomas (Miller y Bodley 1988). Las ribotoxinas se sintetizan como precursores que maduran en los distintos compartimentos celulares. El hongo debe poseer un sistema muy eficiente de reconocimiento de la secuencia señal y de compartimentalización subcelular para que las toxinas sean secretadas únicamente al medio extracelular (Endo et al. 1993, Endo et al. 1993a, Martínez-Ruiz et al. 1998, Lacadena et al. 2007). De hecho, cuando la secuencia señal se altera o se cambia por otra diferente, la expresión heteróloga de restrictocina en *A. nidulans* muestra niveles reducidos de transcripción, mayor lisis celular y deslocalización de la ribotoxina, que no aparece siempre en las fiálides del hongo (Brandhorst y Kenealy 1995), sugiriendo que la secuencia señal original de la restrictocina es altamente eficiente en su papel de proteger al hongo durante la secreción. Además, el gen de la restrictocina en *A. restrictus* posee un intrón al comienzo de su secuencia (Lamy y Davies 1991), lo que estaría en sintonía con el hecho de que, recientemente, un estudio ha demostrado que los intrones pueden utilizarse para atenuar la toxicidad de la barnasa para su uso en terapia génica suicida (Chen 2012). La existencia de este intrón en la restrictocina es sólo un ejemplo de cómo el hongo puede utilizar estrategias adicionales para proteger sus propios ribosomas durante la síntesis y secreción al medio extracelular de las ribotoxinas.

Los potenciales usos biotecnológicos de las ribotoxinas fúngicas.

I. Inmunotoxinas

Durante las últimas dos décadas, las ribotoxinas fúngicas se han estudiado en gran detalle. Su estructura y actividad ribonucleolítica específica se conocen bien en términos moleculares (Lacadena et al. 2007). Y a pesar de que su función en la naturaleza aún no se ha esclarecido, estas ribotoxinas ya se están proponiendo como posibles agentes terapéuticos. De hecho, inicialmente se descubrieron como agentes antitumorales, aunque estudios posteriores revelaran una toxicidad inespecífica frente a células no tumorales (Roga et al. 1971), abandonándose temporalmente su estudio. Más adelante, el interés por las ribotoxinas se recuperó al demostrarse que se podían utilizar como componentes de inmunotoxinas en terapias antitumorales.

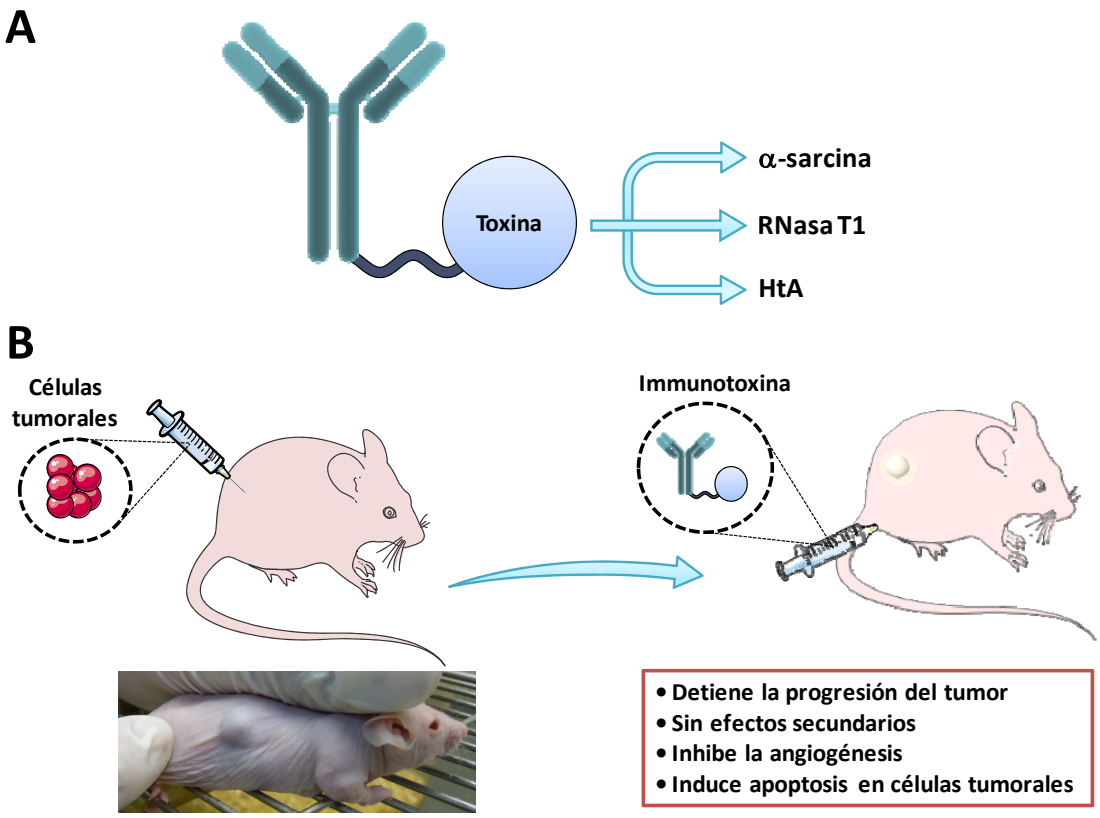

Figura 4. Las ribotoxinas como agentes inmunoterapéuticos. A) Estructura general de una inmunotoxina, que se compone de un dominio de unión, normalmente un fragmento de anticuerpo responsable de interaccionar con un tipo celular específico, y un dominio tóxico, como por ejemplo ribotoxinas, o incluso RNasas no tóxicas como la RNasa T1, que promueven la muerte celular. B) IMTXA33αs, una inmunotoxina que contiene α-sarcina como dominio tóxico, se ha estudiado *in vivo*, demostrándose que es capaz de inhibir el crecimiento tumoral y la angiogénesis en ratones atímicos con xenotrasplantes humanos de cáncer de colon.

Las inmunotoxinas son moléculas quiméricas compuestas por un fragmento de anticuerpo específico, que se encarga de reconocer un tipo celular concreto, unido a un dominio tóxico que promueve la muerte celular (Figura 4) (Reiter y Pastan 1998, Kreitman 2001). Para evitar el elevado tamaño del anticuerpo completo, lo cual podría obstaculizar la penetración de la inmunotoxina en tumores sólidos, se han desarrollado nuevas estrategias que utilizan sólo sus dominios variables. En cuanto al dominio tóxico, se han utilizado varias toxinas como la ricina, la exotoxina A de *Pseudomonas* o la toxina diftérica (Kreitman 2006). A pesar de ser más eficaces, en muchos casos estas toxinas provocan efectos secundarios no deseados.

Las ribotoxinas fúngicas poseen una serie de características que las convierten en candidatas óptimas para la construcción de inmunotoxinas: pequeño tamaño, alta termoestabilidad, poca inmunogenicidad, resistencia a proteasas y alta eficiencia inactivando ribosomas. Ya se han diseñado y caracterizado inmunotoxinas frente a cáncer de colon que contienen ribotoxinas (α-sarcina) y otras RNasas fúngicas (RNasa

T1) como dominio tóxico (Carreras-Sangrà et al. 2012, Tomé-Amat et al. 2012). También se ha desarrollado una inmunotoxina con una variante de HtA incapaz de cruzar las membranas lipídicas, pero que mantiene su actividad ribonucleolítica (Tome-Amat et al. 2015a). Dicha variante sería, por tanto, completamente inocua para las células que no contienen el antígeno específico reconocido por la inmunotoxina, por lo que sería más segura y reduciría los presumibles efectos secundarios derivados de la terapia antitumoral.

La toxicidad de una inmunotoxina depende de muchos aspectos, como la afinidad por el antígeno, la tasa de internalización, el procesamiento intracelular, la liberación de la toxina y la toxicidad intrínseca de ésta. De este modo, el tráfico intracelular del dominio tóxico puede modular los efectos citotóxicos del inmunoconjugado. Por consiguiente, se ha estudiado también dicho tráfico intracelular con distintas inmunotoxinas, que contienen tanto RNasa T1 como diferentes ribotoxinas en su dominio tóxico, mostrando que todas se internalizan a través de endosomas tempranos, siguiendo después diferentes rutas de procesamiento según el dominio tóxico empleado, para finalmente alcanzar el citosol (Tome-Amat et al. 2015a, Tome-Amat et al. 2015b).

Finalmente, se ha ido un paso más allá en el uso terapéutico de estas inmunotoxinas basadas en ribotoxinas, mostrando la eficiencia *in vivo* de la inmunotoxina que contiene α-sarcina como dominio tóxico, IMTXA33αs. Dicha inmunotoxina es capaz de inhibir el crecimiento tumoral y la angiogénesis en ratones atímicos con xenotrasplantes de tumores humanos de cáncer de colon (Figura 4) (Tome-Amat et al. 2015c), un resultado prometedor que muestra el potencial de las ribotoxinas como herramientas terapéuticas frente a patologías tumorales.

II. Implicación en alergias e infecciones por *Aspergillus*.

Algunas especies de *Aspergillus* son patógenos humanos causantes de enfermedades respiratorias tales como asma, aspergilosis broncopulmonar alérgica (ABPA), aspergiloma u otro tipo de infecciones severas, con especial incidencia en pacientes inmunodeprimidos. *A. fumigatus* es el agente etiológico en el 80% de estas enfermedades. La diagnosis e inmunoterapia de alergias producidas por *A. fumigatus* consisten generalmente en la utilización de extractos del hongo, aunque en general dichos extractos son mezclas complejas de cientos de componentes, y por tanto su estandarización es complicada y el riesgo de efectos secundarios anafilácticos es elevado. La mejora en estos diagnósticos y terapias se ha centrado en la producción recombinante de alérgenos. En este contexto es muy reseñable que la ribotoxina Aspf1 sea uno de los principales alérgenos de este hongo (Arruda et al. 1992). A pesar de que no se detecta durante las etapas iniciales de la infección, Aspf1 sí parece jugar un papel importante en procesos alérgicos. De acuerdo con esta idea está la observación de que algunas de las regiones de esta ribotoxina han resultado ser significativamente

alergénicas (Kurup et al. 1998, García-Ortega et al. 2005). Por ello, se han diseñado, producido y caracterizado variantes hipoalergénicas de Aspf1, demostrando que la horquilla β amino-terminal forma parte de uno de los epítopos alergénicos de la ribotoxina (García-Ortega et al. 2005). Además, algunas de estas variantes mutantes no citotóxicas de Aspf1 y α-sarcina son importantes candidatas para su uso en diagnóstico y en terapias inmunomoduladoras de la hipersensibilidad a *Aspergillus*. De hecho, varias de estas variantes se han producido ya en *Lactococcus lactis*, un microorganismo generalmente reconocido como seguro (GRAS, *generally regarded as safe*) que podría utilizarse como vehículo de vacunación en protocolos de inmunoterapia frente a enfermedades relacionadas con Aspf1 (Álvarez-García et al. 2008). Igualmente, se ha establecido un modelo de ratón para la sensibilización frente a *A. fumigatus,* que podría ser muy útil para pruebas pre-clínicas de alérgenos recombinantes y sus derivados (Álvarez-García et al. 2010).

III. Las ribotoxinas como agentes de control de plagas

La probable función insecticida de las ribotoxinas sugiere su posible utilización en el diseño y desarrollo de nuevos biopesticidas. La población mundial crece exponencialmente, y se estima que en el año 2050 alcanzará los 9500 millones de personas. Este rápido aumento de la población requiere de un aumento paralelo de los recursos alimentarios. Las plagas causadas por insectos, nematodos, virus y bacterias, además de la competición con malezas no comestibles, causan el 40% de las pérdidas en la producción agraria mundial cada año. Durante décadas, en el control de plagas se han utilizado insecticidas químicos, como muy bien representa el caso del DDT en los años 40. Este tipo de pesticidas son muy potentes, de rápida acción, baratos de producir y fáciles de diseminar. No obstante, la baja especificidad, su toxicidad y la aparición de resistencias llevaron a muchos países a prohibir, ya en los años 70, el uso de DDT y otros pesticidas químicos. Este problema de la resistencia a pesticidas de origen químico, lejos de aliviarse, ha aumentado con el paso del tiempo. En 1992, más de 500 especies de ácaros e insectos desarrollaron este tipo de resistencias. Además, el coste de descubrir, desarrollar y registrar nuevos pesticidas sintéticos es tan elevado que se ha generado un nuevo interés por métodos alternativos de control biológico. Los biopesticidas se han convertido en un componente importante en el control de plagas respetuoso con el medio ambiente. Su uso, sin embargo, no está todavía muy extendido, constituyendo sólo el 2-3% del mercado de insecticidas (Glare et al. 2012).

El término biopesticida incluye aquellas técnicas de control de plagas que utilizan tanto microorganismos (bacterias, virus, nematodos u hongos) como metabolitos secundarios de estos microorganismos, incluyendo péptidos y proteínas. También comprendería aquellos cultivos modificados genéticamente para mejorar la resistencia al daño causado por insectos, hongos, virus o herbicidas (Carlini y Grossi-de-Sa 2002, Coca et al. 2004).

Los hongos entomopatógenos se han utilizado como agentes de control biológico de ciertas plagas y algunos de ellos incluso se han comercializado, como es el caso de *Bauveria bassiana*, utilizado para controlar la proliferación de numerosos tipos de insectos, o *Metarhizium anisopliae*, recientemente estudiado frente al mosquito *Anopheles gambiae*, transmisor de la malaria (Shaw et al. 2002, Whetstone y Hammock 2007, Kim et al. 2014). *H. thompsonii*, productor de la ribotoxina HtA, es también un conocido hongo entomopatógeno utilizado en el control del ácaro *Varroa destructor*, un ectoparásito de la abeja (Kanga et al. 2002, Peng et al. 2002, Shaw et al. 2002). En algunos casos concretos este hongo parece ser más efectivo incluso que *B. bassiana* y *M. anisopliae* (Rossi-Zalaf and Alves 2006). *H. thompsonii* se registró en 1981 como un micoacaricida (Mycar) para el control del ácaro de los cítricos (McCoy y Couch 1982), pero problemas en su formulación, la falta de persistencia en el campo de cultivo y la baja estabilidad del producto durante el transporte y almacenamiento frenaron su comercialización a finales de los años 80. Por ello, se ha intentado solucionar estos defectos, evaluando nuevas fórmulas que mejoren la distribución, vida media y eficacia en el campo (Sreerama Kumar y Singh 2008)

La manipulación genética se ha utilizado también para aumentar la virulencia de los hongos entomopatógenos en el control de plagas (St Leger y Wang 2010). La primera demostración de la validez de esta aproximación fue la sobreexpresión en el hongo *M. anisopliae* de una proteasa capaz de degradar la cutícula del insecto. Esta técnica también se ha utilizado para ampliar la variedad de especies que puedan ser infectadas. No obstante, ningún organismo modificado genéticamente (OMG) se comercializa actualmente como biopesticida, sobre todo debido a las regulaciones estrictas y la imagen pública tan negativa que existe respecto a este tipo de organismos (Glare et al. 2012).

Algunos biopesticidas exitosos se basan en el uso de compuestos producidos por microorganismos, en lugar de provocar la infección utilizando el organismo completo (Glare et al. 2012). Por ello, entender el mecanismo de infección de estos hongos, así como los factores de virulencia implicados, podría contribuir al desarrollo de nuevas fórmulas para el control de plagas. En este sentido, el aislamento y caracterización de ribotoxinas fúngicas y su actividad contra insectos abre la posibilidad de utilizarlas precisamente como bioinsecticidas, de manera independiente, o combinados con otros compuestos (Olombrada et al. 2013, Olombrada et al. 2014). La potencial toxicidad de las ribotoxinas frente a vertebrados se podría solucionar utilizando diferentes formas mutantes (Herrero-Galán et al. 2012, Tome-Amat et al. 2015a) que mantendrían su actividad ribonucleolítica frente a los ribosomas, pero serían incapaces de atravesar la membrana de las células de mamífero, disminuyendo así su citotoxicidad inespecífica.

Debido a su actividad insecticida intrínseca, los virus patógenos de insectos o baculovirus representan otra estrategia alternativa en el control de plagas (Figura 5). Los baculovirus naturales ya se han utilizado como biopesticidas, aunque las versiones

recombinantes generadas por ingeniería genética parecen ser una mejor alternativa (Cory et al. 1994, Inceoglu et al. 2001) dado que pueden acelerar la muerte del insecto sin conferir ninguna ventaja ecológica sobre los baculovirus silvestres. Los baculovirus tienen además la ventaja adicional de representar una fórmula sencilla y estable con gran especificidad por el hospedador. Un ejemplo es el diseño de un baculovirus mejorado que expresa una proteína de *A. fumigatus* (Gramkow et al. 2010) capaz de inducir la muerte más rápido que el virus silvestre en larvas de *S. frugiperda*. Estos resultados animan al desarrollo de herramientas similares utilizando las ribotoxinas. Estos baculovirus diseñados para la producción de ribotoxinas serían específicos de insectos, minimizando así los efectos secundarios en otros organismos. Además, el extenso conocimiento acumulado durante todos estos años acerca de las ribotoxinas puede ser de inestimable ayuda en el diseño y producción de toxinas insecticidas más eficientes. El único problema relacionado con el uso de baculovirus en el control de plagas reside en que la opinión pública no es, en general, favorable a la introducción de virus modificados genéticamente. Pese a todo, ya se están produciendo baculovirus respetuosos con el medio ambiente, diseñados para que los virus vayan perdiendo su actividad a medida que se suceden los ciclos infectivos y terminen revertiendo al fenotipo silvestre tras un periodo razonable de tiempo (Shim et al. 2013).

IV. El estudio de las enfermedades relacionadas con el ribosoma. La biogénesis del ribosoma como diana adicional de las ribotoxinas.

Los ribosomas son las máquinas moleculares más finamente construidas y reguladas en la célula. Son esenciales para la vida, produciendo todas las proteínas necesarias para el crecimiento celular. En humanos, cuando se alteran los componentes ribosomales, o las moléculas involucradas en el control de su correcta estructura y función, aparecen un número de enfermedades heterogéneas, denominadas ribosomopatías. Estas enfermedades tienen en común una disfunción ribosomal, pero difieren en el mecanismo por el que se producen, los síntomas que se manifiestan y los posibles tratamientos a aplicar (Tabla 1). Algunos desórdenes como la anemia de Diamond-Blackfan (DBA), por ejemplo, se han relacionado con mutaciones en proteínas ribosomales, mientras que otras, como el síndrome de Schwachman-Diamond (SDS) o el síndrome de Treacher-Collins (TCS) están directamente relacionadas con un defecto en el propio proceso de biogénesis del ribosoma (Narla y Ebert 2010, Nakhoul et al. 2014, Ruggero y Shimamura 2014, Warner 2015). Entre las anomalías que presentan estas enfermedades destacan fallos en la médula ósea, defectos del desarrollo y un elevado riesgo de padecer cáncer, aunque los síntomas son muy diversos, incluso entre pacientes con la misma enfermedad (Narla y Ebert 2010). Existen, por ello, todavía muchas cuestiones sin resolver acerca de las ribosomopatías. Entre otras, resulta llamativo cómo siendo el ribosoma una maquinaria tan conservada en todas las células, mutaciones en las proteínas ribosomales son capaces de causar fenotipos tan variados. Mutaciones que, a veces,

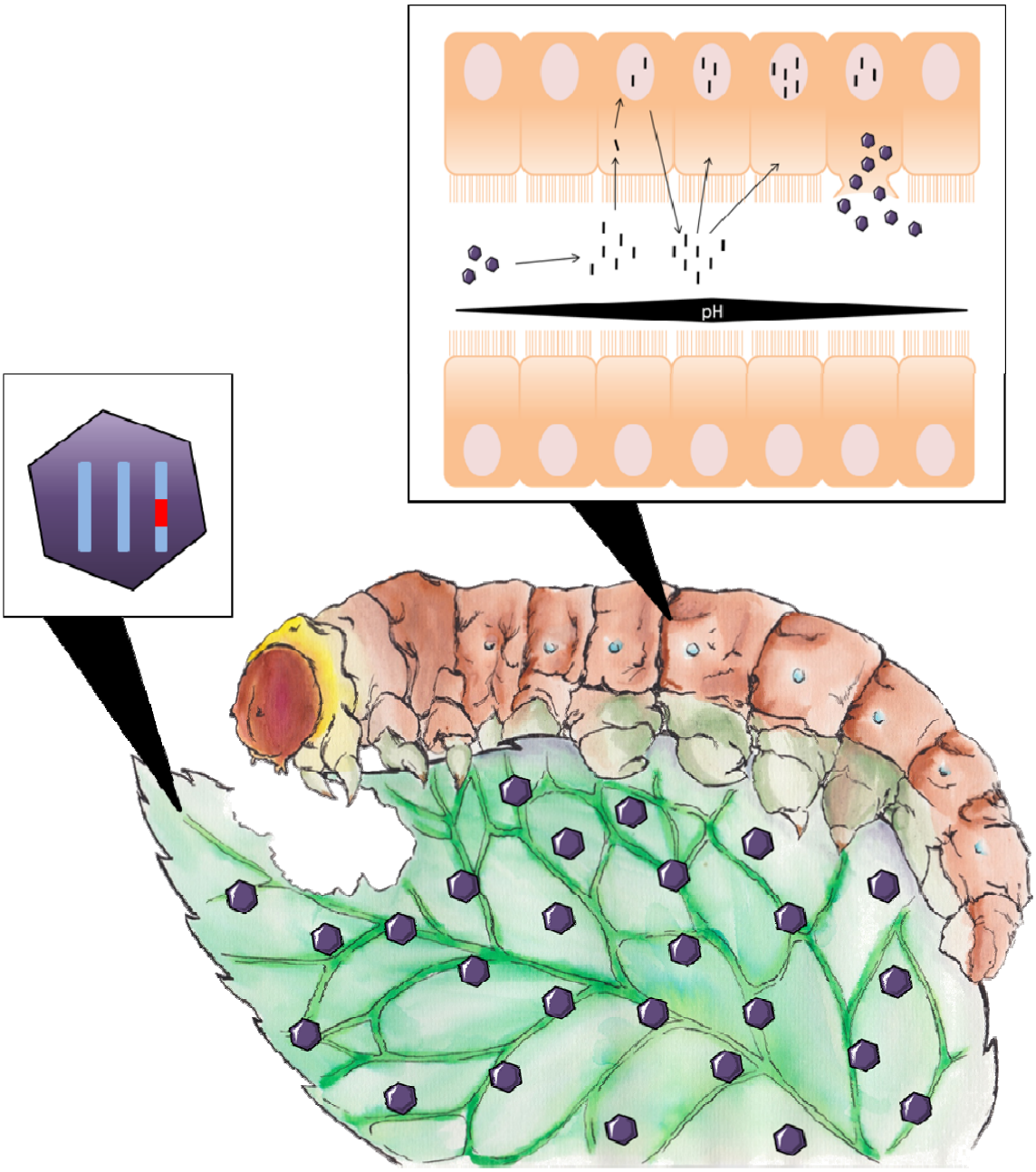

Figura 5. Baculovirus como biopesticidas. Baculovirus modificados genéticamente que contienen genes para toxinas específicas de insecto, hormonas u otras enzimas se rocían sobre el follaje de la planta de modo que son ingeridos por las larvas o los insectos adultos. Las estructuras poliédricas que los componen se solubilizan en el tracto digestivo debido a un aumento del pH en esta región, y se liberan viriones que empiezan a replicarse dentro del núcleo de las células epiteliales, produciendo más viriones que se liberan individualmente (estadíos tempranos de la infección) o agrupados en forma de poliedros (estadíos tardíos de la infección). Paralelamente a la infección por el virus, se expresaría la toxina o molécula insecticida, que aceleraría la muerte del insecto.

incluso afectan a tipos celulares o tejidos específicos. Algunas explicaciones plausibles para esta especificidad de tejido podrían incluir que a) en tejidos de rápida proliferación, las células son más sensibles a mutaciones que disminuyan el número de

ribosomas, y por tanto se ven más afectados que los tejidos o células que se dividen más despacio, o b) la composición de los ribosomas puede ser diferente en distintos tipos celulares, de modo que la reducción en los niveles de una proteína ribosomal puede afectar de manera diferente a los niveles de mRNA traducido (McCann y Baserga 2013, Nakhoul et al. 2014). El esclarecimiento de las bases de estas ribosomopatías requiere de un conocimiento más profundo del ribosoma, y muy especialmente de su biogénesis, ya que parece que este proceso se ve afectado en un número relativamente elevado de estas enfermedades. Por suerte, la levadura *S. cerevisiae* parece ser un sencillo a la vez que buen organismo modelo para el estudio de estos procesos patológicos (Menne et al. 2007, Woolford y Baserga 2013).

La biogénesis del ribosoma es un proceso celular muy complejo a través del cual las subunidades del ribosoma se ensamblan en una unidad funcional. En eucariotas, este proceso comienza en el nucléolo. Seguidamente, ambas subunidades van madurando a medida que se desplazan por el nucleoplasma y el citosol (Figura 6). Más de 300 factores, incluidos proteínas y RNAs, participan en la producción del ribosoma eucariota en la levadura *S. cerevisiae*. En humanos este número es aún mayor, en concordancia con la mayor complejidad y tamaño de nuestro ribosoma (Lafontaine 2015, Warner 2015). Este complejo proceso de maduración comienza en la levadura con la transcripición de los pre-rRNA 5S y 35S por parte de las RNA polimerasas I y III. Estos pre-rRNAs sufren modificaciones post-transcripcionales y se asocian a proteínas ribosomales y a otros factores adicionales para formar la partícula 90S. Dicha partícula se escinde, dando lugar a las subunidades pre-40S y pre-60S, que a partir de ese momento siguen distintas rutas de maduración. Las subunidades pre-40S se exportan rápidamente al citosol tras sufrir relativamente pocos cambios y, una vez allí, se lleva a cabo el procesamiento del pre-rRNA 20S a rRNA 18S maduro. La maduración de la subunidad pre-60S es, por el contrario, mucho más compleja y conlleva varias etapas de maduración dentro del propio nucleo. Una vez en el citosol, ya sólo quedan unos pocos factores proteicos unidos a la subunidad pre-60S, que se van separando de ella de manera secuencial. Entre otros eventos, destacan la liberación de factores asociados al túnel del ribosoma, el ensamblaje del tallo ribosómico (proteínas P0, P1 y P2 del ribosoma) y la liberación del factor Tif6 (eIF6 en humanos) por acción de Sdo1 y la GTPasa Efl1, siendo Tif6 un factor que previene la asociación prematura de ambas subunidades (Menne et al. 2007, Kemmler et al. 2009, Lo et al. 2009, Panse y Johnson 2010, Woolford y Baserga 2013, Gerhardy et al. 2014). La aparición de mutaciones en el ortólogo humano de Sdo1, SBDS, son los causantes de la mayoría de casos de síndrome de Shwachman-Diamond (Brina et al. 2014, Ruggero y Shimamura 2014).

Gracias a metodologías complejas, como la genética de levaduras y la purificación de afinidad en tándem (TAP, *Tandem Affinity Purification*) combinada con la espectrometría de masas, se ha avanzado significativamente en el conocimiento de la biogénesis del ribosoma durante los últimos 20 años. No obstante, aún quedan

Tabla 1.- Lista de defectos genéticos asociados a ribosomopatías (adaptado de Freed et al. 2010).

DEFECTO GENÉTICO	FUNCIÓN ALTERADA	ENFERMEDAD	CARACTERÍSTICAS	TRATAMIENTO	REFERENCIAS
RPS19, RPS26, RPL5, RPL11 y otras proteínas ribosomales.	Diferentes etapas del procesamiento de pre-rRNAs	Anemia de Diamond Blackfan (DBA)	Anemia, fallos en la médula ósea, retraso del crecimiento, anomalías congénitas, defectos cardíacos, predisposición al cáncer.	Corticoesteroides, transfusiones, trasplante de células madre hematopoyéticas	Draptchinskaia *et al.* 1999, Cmejla *et al.* 2009 Farrar *et al.* 2008, Gazda *et al.* 2008, Gazda y Sieff 2006;Horos y von Lindern 2012;Lipton y Ellis 2009
RPS14	Procesamiento del pre-rRNA 18S	Síndrome 5q	Anemia macrocítica severa, predisposición a padecer cáncer	Lenalidomida	Ebert *et al.* 2008, Padron *et al.* 2011
SBDS	Maduración y transporte al citoplasma de la subunidad ribosomal 60S	Síndrome de Shwachman-Diamond (SDS)	Insuficiencia pancreática severa, defectos hematológicos, anomalías esqueléticas, predisposición a padecer cáncer	Enzimas pancreáticas, trasplante de células madre hematopoyéticas	Boocock *et al.* 2003, Myers *et al.* 2013; Rujkijyanont *et al.* 2009
DKC1	Deficiencia en la telomerasa, agravada por defectos en la pseudouridilación de snoRNP de la caja H/ACA	Disqueratosis congénita asociada al X	Anomalias mucocutáneas, fibrosis pulmonar, fallo de la médula ósea, predisposición a padecer cáncer	Oxymetolona, trasplante de células madre hematopoyéticas	Heiss *et al.* 1998, Mason y Bessler 2011; Walne y Dokal 2009
RMRP	Maduración del rRNA 5.8S; degradación de RNAs regulados por el ciclo celular.	Hipoplasia cartílago cabello (CHH)	Corta estatura, hipoplasia capilar, anomalías óseas, predisposición al cáncer	Sintomático	Martin y Li 2007; Ridanpaa *et al.* 2001
TCOF1	Transcripción de rDNA y metilación del rRNA 18S	Síndrome de Treacher-Collins (TCS)	Anomalías craneofaciales	Sintomático	Gonzales *et al.* 2005; Schlump *et al.* 2012; Group 1996
EMG1	Maduración de la subunidad ribosomal 40S	Síndrome de Bowen-Conradi	Grave retraso del crecimiento	Ninguno	Armistead *et al.* 2009, Meyer *et al.* 2011, Wurm *et al.* 2010
hUTP4/Cirhin	Maduración del rRNA 18S	Cirrosis infantil de los indios de Norteamérica	Cirrosis biliar, letal en la adolescencia si no se realiza untrasplante	Trasplante de hígado	Chagnon *et al.* 2002, Freed *et al.* 2010; Freed *et al.* 2012

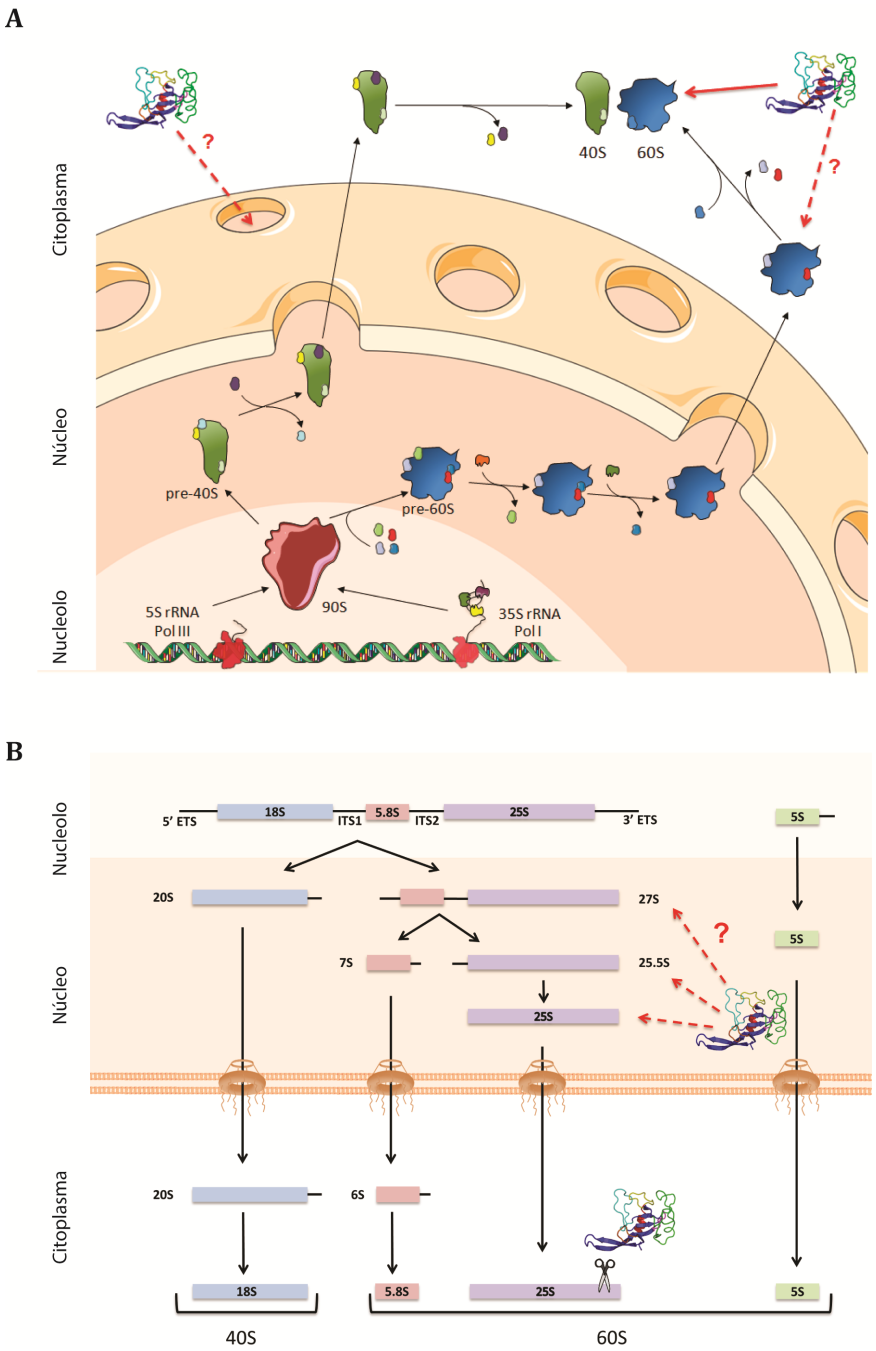

Figura 6. A) Representación esquemática de la maduración del ribosoma, que comienza cuando las RNA polimerasas I y III transcriben los pre-rRNAs 35S y 5S, respectivamente. Estos rRNAs se asocian a proteínas ribosomales, y a otros factores, dando lugar a la partícula 90S. Tras el corte del pre-rRNA 35S, la partícula 90S se escinde en pre-60S (azul) y pre-40S (verde), las cuales siguen vías de procesamiento diferentes desde ese momento. La maduración está dirigida por factores de ensamblaje a través del nucleoplasma. Ya en el citosol, las últimas etapas de maduración dan lugar a subunidades ribosomales listas para comenzar la traducción. Las ribotoxinas atacan subunidades 60S maduras, pero su toxicidad podría extenderse también a partículas pre-60S e incluso al resto de la ruta de biogénesis del ribosoma. B) Procesamiento de pre-rRNAs (simplificada de Gerhardy et al. 2014). El precursor común 35S se escinde, originando pre-rRNA 20S, que es procesado en el citosol y da lugar al rRNA 18S maduro, y pre-rRNA 27S, que se procesa en el nucleoplasma y el citosol dando lugar a las formas maduras 25S y 5.8S. El pre-rRNA 5S es procesado en el núcleo. Las ribotoxinas podrían afectar esta ruta de procesamiento cortando no solo el SRL en rRNA maduros sino también en formas pre-rRNA.

muchas cuestiones sin resolver. Las ribotoxinas fúngicas podrían ayudar a responder algunas de ellas funcionando como herramientas para analizar la ruta de maduración de las partículas pre-60S, especialmente en el citosol, ya que su diana, el SRL, se localiza en una zona importante para la maduración del ribosoma. Desde este punto de vista, sería interesante saber si estas ribotoxinas pueden acceder al núcleo y, si lo hacen, si son capaces de cortar el SRL de las partículas pre-60S. Así, el comportamiento de las ribotoxinas sobre partículas pre-ribosómicas puede contribuir al conocimiento de las rutas de procesamiento y plegamiento de los pre-rRNAs en el campo de la biogénesis del ribosoma, así como a la resolución del papel y localización de algunos factores proteicos que participan en el proceso. A largo plazo, por tanto, estos estudios podrían contribuir al entendimiento de patologías como el mencionado síndrome de Schwachman-Diamond u otras ribosomopatías relacionadas.

Conclusiones y futuras perspectivas

Durante las últimas décadas, se han estudiado con detalle la estructura y la función de las ribotoxinas fúngicas. A día de hoy, todo el conocimiento acumulado sobre esta familia de toxinas ha permitido su utilización en diferentes aplicaciones, como el diseño de inmunotoxinas específicas frente a cáncer de colon o el establecimiento de un modelo murino para el estudio de la aspergilosis. El descubrimiento de la ribotoxina HtA ha planteado la posibilidad de que en la naturaleza las ribotoxinas tengan una función defensiva con actividad insecticida. Su elevada eficacia frente a larvas y células de insecto las hace buenas candidatas para el desarrollo de nuevos biopesticidas. Por otra parte, el elevado conocimiento de la estructura y función de las ribotoxinas podría ser utilizado para estudiar la biogénesis del ribosoma y, a largo plazo, contribuir al estudio de diversas ribosomopatías. En conclusión, aunque aún queda mucho trabajo por realizar, las ribotoxinas fúngicas poseen un gran potencial para ser utilizadas como herramientas biotecnológicas en numerosas aplicaciones.

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Fungal ribotoxins as biotechnological tools. A variety of multidisciplinary applications.

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Abstract

Fungi establish a complex network of biological interactions with other organisms in nature. In many cases, these involve the production of toxins for survival or colonization purposes. Some of these toxins have been shown to display high insecticidal activity, which has led to the idea that they could play a role in fungi defense against predators. Among these toxins, ribotoxins stand out as promising candidates for their use in biotechnological applications. They constitute a group of highly specific extracellular RNases that target a universally conserved sequence of rRNA in the ribosome, the sarcin-ricin loop. Moreover, their biotechnological potential has already been verified since they are highly efficient as antitumoral agents when used as part of inmunotoxins. This review deals with the insecticidal role of fungal ribotoxins in nature as well as their potential use as biotechnological tools in different applications, from therapeutic uses in cancer or allergy to their use as bioinsecticides for pest control or as tools for the study of the basis of some human ribosomopathies.

Keywords: fungal ribotoxins, RNases, ribosome, SRL, insecticide, biopesticide, immunotoxin, cancer, ribosomopathies.

Abreviations: RNase, ribonuclease; SRL, sarcin-ricin loop; RIP, ribosome-inactivating protein; HtA, hirsutellin A; rRNA, ribosomal RNA; GM, genetically modified; DDT, dichlorodiphenyltrichloroethane.

Introduction

Organisms populate almost any ecological niche of the planet and establish a high variety of biological interactions, ranging from mutualism to antagonism, mainly in search for resources to survive (light, nutrients, water, etc.). In particular, fungi constitute a rich source of nitrogen and phosphorous for arthropods and are under

constant attack by fungivorous such as collembolan, mites and insects (Berenbaum and Eisner 2008). However, they can also present mutualistic relationships like those established with fungus farming ants. Thus, a complex network of interactions is established in nature involving preying, defense and feeding. In this context, fungi produce a great variety of toxins, many of which are proteins. These toxins are secreted with many different purposes, although self-defense and fungi development have been suggested to be their principal functions.

Fungal ribotoxins are a family of extracellular ribonucleases that have been studied in deep detail since their discovery in the early 1960's. They are not the only extracellular RNases produced by fungi, but are the only ones shown to display cytotoxic activity. Ribotoxins are produced by several fungi species, mostly from the genus *Aspergillus*, although other entomopathogenic fungi such as *Hirsutella thompsonii* also produce them (Martínez-Ruiz et al. 1999a, Martínez-Ruiz et al. 1999b, Lacadena et al. 2007, Herrero-Galán et al. 2008). They are highly specific, exerting their ribonucleolytic activity on the larger molecule of rRNA of the ribosome. From this perspective, they could be included in the group of ribosome-inactivating proteins (RIPs), however, this term usually refers to those toxins with N-glycosidase activity and plant origin, best represented by ricin, a toxin found in the seeds of the castor bean plant *Ricinus communis*. Both RIPs and ribotoxins target the same universally conserved sequence of rRNA, the so-called sarcin-ricin loop (SRL), by either depurinating a single nucleotide or cleaving a unique phosphodiester bond, respectively. However, the mechanisms by which both types of toxins reach their target are quite different (Chiou et al. 2008, Olombrada et al. 2014).

Even though the biological function of ribotoxins has not been cleared up yet, several studies have shown the insecticidal properties of at least two of these proteins, supporting their involvement in defensive and parasitism mechanisms (Brandhorst et al. 1996, Brandhorst et al. 2001, Olombrada et al. 2013). The detailed study of the structure and function of this family of toxic proteins has highlighted their potential use in biotechnological applications, either as bioinsecticides to control insect pests (Olombrada et al. 2014a) or their use in human therapies as antitumoral agents (Tome-Amat et al. 2015c). Moreover, they could also be used as specific tools for the study of human ribosome-related diseases, the so-called ribosomopathies (Narla and Ebert 2010, Nakhoul et al. 2014).

Fungal ribotoxins

Ribotoxins are not the only RNases produced by fungi. Indeed, they are part of a much wider group of fungal extracellular RNases, being ribotoxins the only ones known to show cytotoxic activity. Ribotoxins are larger than non-toxic fungal RNases but still small proteins of about 150 amino acids, and generally basic proteins. The main structural difference between non-toxic RNases, such as RNases T1 or U2, and

ribotoxins would be that the latter contain longer and positively charged loops, which have been suggested to be the structural basis of their toxicity (Martínez-del-Pozo et al. 1988). Identification of these structural features that made ribotoxins such efficient natural killers would be a major step towards their utilization as specifically targeted weapons against insect pests or different human pathologies. So far, the best characterized ribotoxins are α-sarcin, restrictocin, Aspf1, and hirsutellin A (HtA). The first three of these proteins show a high degree of conservation with sequence identities above 85%. However, HtA, a ribotoxin produced by the entomopathogen fungi *Hirsutella thompsonii,* only shares 25% of sequence identity with them (Fig. 1). This observation confirmed that the presence of ribotoxins among fungi is more widespread than initially considered, and not restricted to the genus *Aspergillus* (Liu et al. 1995, Martínez-Ruiz et al. 1999a, Martínez-Ruiz et al. 1999b, Varga and Samson 2008).

The ribonucleolytic activity of ribotoxins is extremely specific, targeting a highly conserved sequence of rRNA in any ribosome known, the SRL (Fig. 1). This sequence is located on the larger molecule of rRNA of the large ribosomal subunit. During translation, the SRL together with the uL11-binding region, the L7/L12 stalk (P1/P2 in yeast) and ribosomal proteins uL6 and uL14, constitute an elongation factor-binding site that is required for correct functioning of the ribosome (García-Ortega et al. 2010, Voorhees et al. 2010). The SRL is the longest known universally conserved rRNA sequence, and therefore its structure is highly preserved in archaeal, bacterial and eukaryotic ribosomes. It consists of a distorted hairpin, and the most significant structural features are a GAGA tetraloop and a bulged G motif. Ribotoxins cleave a single phosphodiester bond of the GAGA tetraloop, producing a small fragment of 300-400 nucleotides of rRNA, known as the α-fragment. They are cyclizing RNases displaying the general acid-base type endonucleolytic cleavage of RNA mechanism in two steps, just like other members of the RNase T1 family (Lacadena et al. 1998, Lacadena et al. 2007). Cleavage of this phosphodiester bond inhibits protein biosynthesis and produces cell death by apoptosis (Olmo et al. 2001).

The three-dimensional structure of several ribotoxins is known at atomic resolution (Fig. 1) (Yang and Moffat 1996, Pérez-Cañadillas et al. 2000, Viegas et al. 2009), and mutational analyses have allowed the assignment of specific roles to different residues (implication in the catalysis, ribosome and SRL recognition, interaction with the cell membrane, etc.). In the case of α-sarcin, the catalytic residues are His 50, Glu 96 and His 137. Glu 96 would act as a general base and His 137 as a general acid (Lacadena et al. 1995, Martinez-Ruiz et al. 1999). His 50 would contribute to the stabilization of the transition state. These three residues are required for the specific inactivation of ribosomes. Additional mutational studies showed that Tyr 48, Arg 121 and Leu 145, although not essential, would also contribute to the ribotoxin activity of α-sarcin. The equivalent residues in HtA (His 42, Glu 66 and His 113) were

identified by comparison of its structure with that of other ribotoxins, showing that the essential residues are conserved, but also presenting other features closer to the T1-

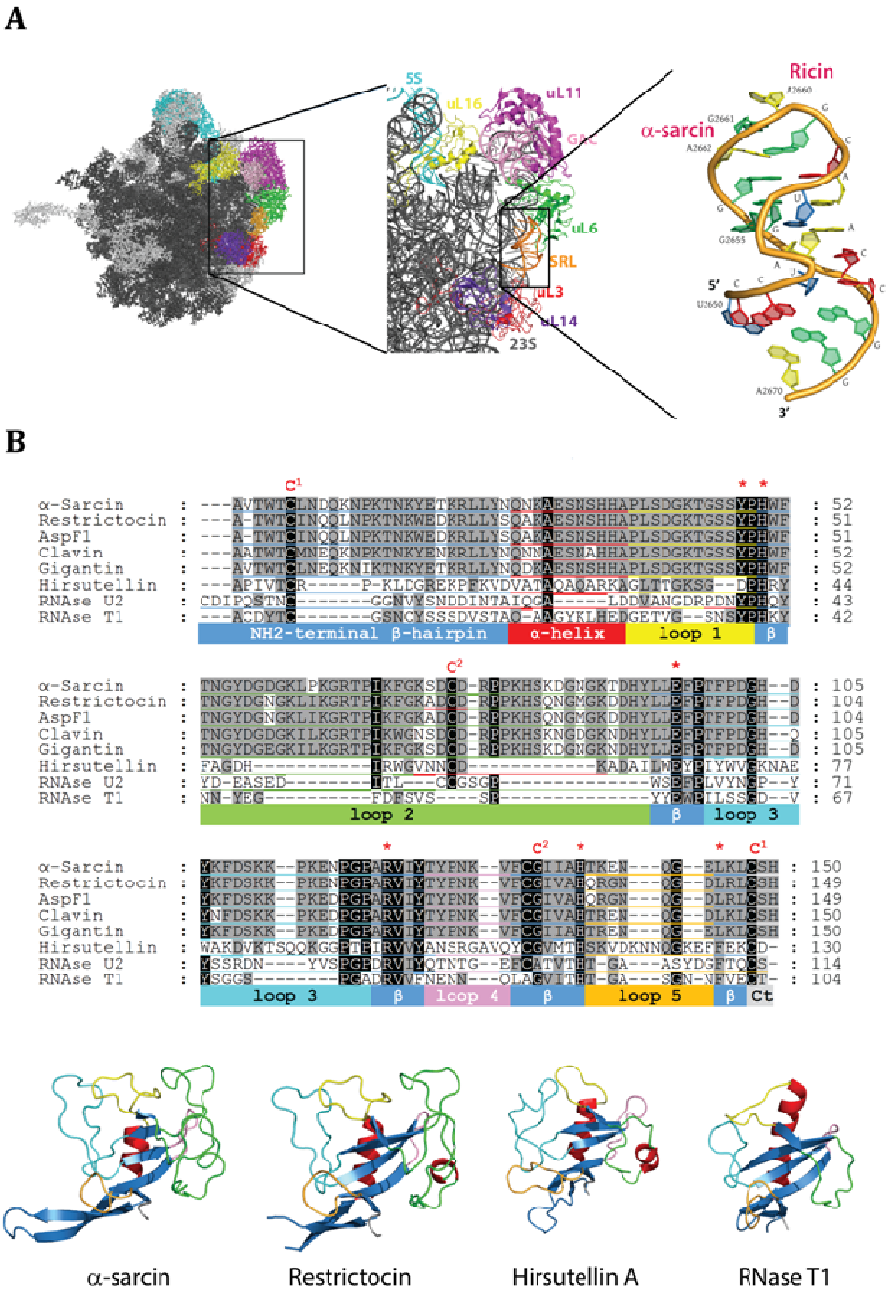

Figure 1.- A) Three-dimensional structure of the large ribosomal subunit of *Escherichia coli* (PDB ID: 2AW4). Conserved proteins around the SRL (orange) region appear in different colors: uL3 (red), uL6 (green), uL11 (pink), uL14 (purple), and uL16 (yellow). rRNA 23S (gray) and 5S (cyan) are also shown. GAC is for GTPase-activated-center. Two different close ups are detailed including the SRL structure showing the bond cleaved by α-sarcin (green) and the adenine depurinated by ricin. B) Sequence alignment of most representative fungal RNases from the RNase T1 family. Conserved Cys forming disulphide bridges (C) as well as the active site residues (*) are indicated. Conserved amino acids (light grey boxes) in at least seven sequences are highlighted in black. Elements of secondary structure are displayed by color as indicated in the boxes. The three dimensional structures of RNase T1 and the three most representative ribotoxins are also shown (PDB IDs: 1DE3, 1AQZ, 2KAA, 9RNT). Diagrams were generated with PyMOL.

like RNases (Phe 126 instead of the equivalent Leu 145, for example) or even some other ones which were completely new (Asp 40 instead of Tyr 48) (Herrero-Galán et al. 2012a). Mutagenesis studies have indeed revealed that the active site of HtA would provide a more adaptable microenvironment than the one described for α-sarcin since none of the three identified catalytic residues seemed to be indispensable for catalysis as was the case for α-sarcin (Herrero-Galán et al. 2012a).

The toxicity of ribotoxins results from the combination of their highly specific RNase activity and their ability to cross membranes. So far no protein receptor for ribotoxins has been found in the cell. For that reason, even though ribotoxins can potentially inactivate any ribosome, the lipid composition of cell membranes plays an important role in their cytotoxic specificity. Accordingly, it has been described how ribotoxins are more efficient on transformed or virus-infected cells, most likely due to altered permeability and/or composition of the cell membrane (Turnay et al. 1993, Olmo et al. 2001) Moreover, insect cells have a particular lipid composition in their membranes; as a consequence, they are thinner and more fluid, making them more sensitive and accessible to ribotoxins activity (Marheineke et al. 1998, Olombrada et al. 2013). The use of lipid model systems has proven that α-sarcin interacts with lipid vesicles rich in acidic phospholipids. This interaction then causes vesicle aggregation followed by fusion and intermixing of vesicles' phospholipids and, finally, leakage of their aqueous contents (Fig. 2) (Gasset et al. 1989, Gasset et al. 1990).

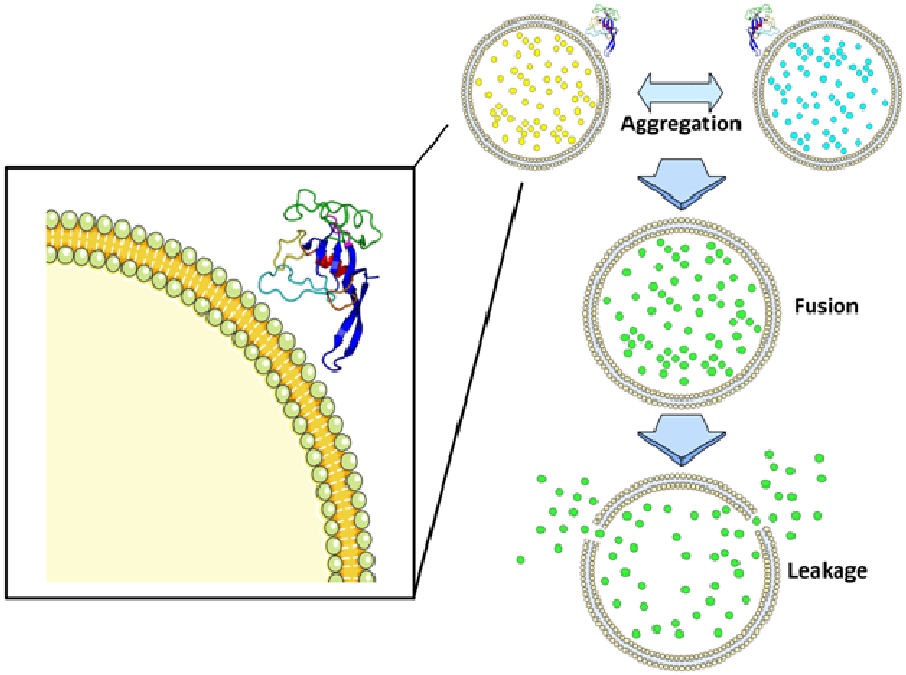

Figure 2.- Scheme showing the lipid fusion mechanism induced by α-sarcin. This fusion results in leakage when isolated model lipid vesicles are employed.

However, this does not seem to be a cell internalization mechanism conserved among ribotoxins, since HtA does not promote vesicle aggregation even though it shows a higher membrane-permeabilizing ability than α-sarcin, at least in leakage experiments (Herrero-Galán et al. 2008, Olombrada et al. 2013). In α-sarcin, the region comprising residues 116-139 was thought to participate in the hydrophobic interaction with membranes (Mancheño et al. 1995, Mancheño et al. 1998) and recently it was proposed that Lys residues 111 and 114 would also take part in the electrostatic interactions needed to bring the vesicles into contact (Castaño-Rodríguez et al. 2015). For HtA, a role in membrane-permeabilizing activity has been proposed for residues Trp71 and Trp 78 (Herrero-Galán et al. 2012b). The ability to interact with lipid membranes has also been associated with the N-terminal β-hairpin of ribotoxins. Deletion of this positively charged region in α-sarcin produces a non-toxic but active ribonuclease with altered membrane interaction properties (García-Ortega et al. 2001). It is in this region where HtA and restrictocin show more variability when compared to α-sarcin. In HtA, the N-terminal β-hairpin is much shorter (Fig. 1), but this truncation might be compensated by the extension of loop 5, which also exhibits a higher amount of positive charges (Herrero-Galán et al. 2012b).

The positively charged surface of ribotoxins may allow them to establish favorable electrostatic interactions with the ribosome (García-Mayoral et al. 2005, Korennykh et al. 2006, Álvarez-García et al. 2009). Moreover, the highly specificity of ribotoxins requires of additional elements besides the active site capable of interacting with the ribosome and recognize the SRL. So far, several regions have been proposed to participate in the specific recognition of the SRL and other ribosome elements. The Lys-rich region of loop 3 would interact with a phosphodiester bond around the bulged G of the SRL, whereas residues 51-55 of loop 2 and some residues of loop 5 would contact the GAGA tetraloop that is cleaved by the toxin (Yang et al. 2001, García-Mayoral et al. 2005). Docking models using the *Haloarcula marismortui* ribosome and α-sarcin structures suggested that apart from potential interactions with the SRL, α-sarcin may interact with neighbouring ribosomal proteins. A short sequence of loop 2 would make contact with ribosomal protein uL6 and the N-terminal β-hairpin, specifically the stretch 11-16, would interact with ribosomal protein uL14 (Fig. 1). Moreover, residues 11-16 of α-sarcin show homology with a region found in the elongation factor 2 (EF-2) from *Saccharomyces cerevisiae* (García-Mayoral et al. 2005). Based on these observations, other ribosomal regions involved in the recruiting of elongation factors during translation could establish some kind of interactions with toxins. That is the case of the ribosomal stalk proteins and RIPs like ricin, trichosantin and Shiga-like toxins (Chiou et al. 2008, Tumer and Li 2012). This protruding structure of the ribosome serves as an anchoring platform for several RIPs to further recognize their target, the SRL. In spite of sharing the same target, not all RIPs behave identically, since no interaction has been found between pokeweed antiviral protein and the eukaryotic stalk proteins (Ayub et al. 2008). In the case of ribotoxins, the eukaryotic

ribosomal stalk does not seem to participate; at least in α-sarcin inactivation of the ribosome (Olombrada et al. 2014). This result suggests that ribotoxins use a different mechanism of ribosome recognition than that used by RIPs like ricin.

The genus A*spergillus* and other ribotoxin-producer fungi.

Fungal ribotoxins were first discovered in the 1960's during a screening program searching for antibiotics and antitumor agents. The mold *Aspergillus giganteus* produced a protein that could inhibit sarcoma and carcinoma induced in mice (Olson and Goerner 1965) which was named α-sarcin. Soon after, two other antitumor proteins with similar activity, restrictocin and mitogillin, were discovered in *Aspergillus restrictus*. Recent studies proved that all species assigned to *Aspergillus* section *Clavati* contain ribotoxin genes (Varga and Samson 2008). Aspergilli are a ubiquitous and complex group of filamentous fungi containing more than 185 species, including human pathogens, like *Aspergillus fumigatus,* as well as others used in industry for the production of food and enzymes, like *Aspergillus oryzae* (*Machida et al. 2005)*. The genome sequence of these two species along with that one of *Aspergillus nidulans* was completed not too many years ago (Galagan et al. 2005). Interestingly, *A. fumigatus*, an opportunistic human pathogen, produces a ribotoxin, AspF1 (Fig. 1). By 2008, already seven species of *Aspergillus* had their genome sequenced. This was a promising step towards the understanding of fungi biology and evolution. Indeed, sequencing the genome of other fungi could also be used for the identification of new species that produce ribotoxins. Moreover, the comparison of these genomes with those from non-producer species could, therefore, shed new light on what could be the biological function of these proteins.

Most filamentous fungi have a complex life cycle. Like other Ascomycota, some Aspergilli can have both sexual and asexual reproductive cycles (Fig. 3). *A. nidulans* constitutes a good example to describe them since it can present both types of reproduction (Casselton and Zolan 2002). In the sexual reproduction, karyogamy (nuclei fusion) and meiosis take place. The fungus develops a fruiting body, the cleistothecium, which produces sexual spores or ascospores. So far, only a few Aspergilli species have been shown to have sexual cycles. During the asexual cycle, the mycelium, which develops from a single haploid spore, differentiates into many identical spores named conidia or conidiospores, which can form a new web of hyphae. There are only a few studies of ribotoxin expression by their natural hosts and its role in its life cycle. In those, the presence of ribotoxins has been detected during the asexual reproduction of *Aspergillus restrictus*. The ribotoxin restrictocin, which is almost indistinguishable from α-sarcin, is produced by *A. restrictus* at a very specific moment of fungal development: the initial steps of conidiophore formation (Brandhorst and Kenealy 1992). First studies in liquid cultures where the fungus is immersed in the medium showed a correlation between the appearance of restrictocin

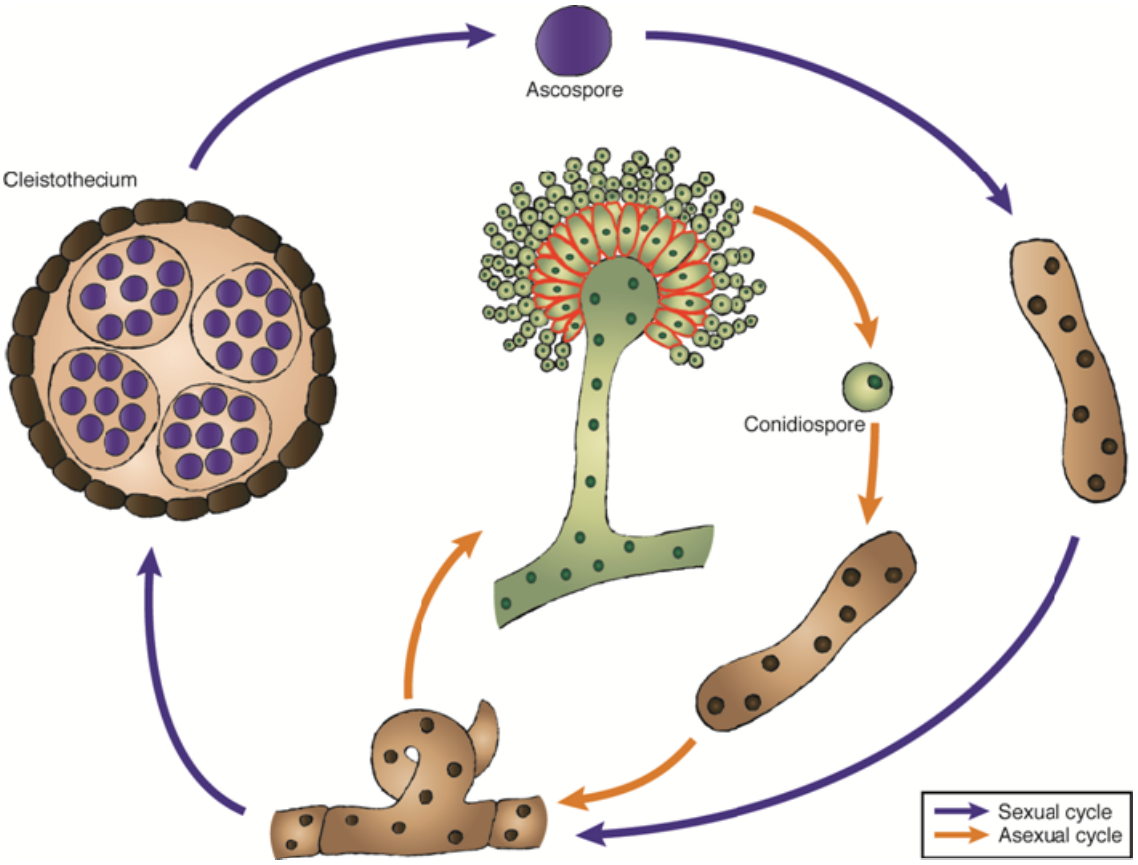

Figure 3.- Life cycle of *Aspergillus* and suggested localization of ribotoxins. *Aspergillus* can enter a sexual or asexual reproductive cycle. During the sexual cycle, the mycelium forms a fruiting body, the cleistothecium, which holds the ascospores that once released into the environment can form a new mycelium. In the asexual reproduction, the mycelium differentiates into identical asexual spores or conidiospores. Ribotoxins would be produced during maturation of the conidia, and would be located on the edge of the phialides (shown in red in the figure). Additionally, a parasexual cycle can take place in *Aspergillus* (not shown).

and differentiated structures (conodiophores) (Yang and Kenealy 1992). Yet, these cell structures do not develop properly in a liquid environment. Further studies in solid medium, which resembles better the natural environment of *A. restrictus*, normally found in water-stressed environments, revealed that restrictocin levels increased until the time of conidiation and was apparently degraded afterwards (Brandhorst and Kenealy 1992). Furthermore, in solid medium restrictocin remained attached exclusively to the phialides and disappeared after conidia maturation (Fig. 3). Plausible explanations for this pattern in the ribotoxin expression could be that restrictocin has a role in the process of conidiation, or that restrictocin has just a protective function of the maturing conidia from predators until the development is complete. Once conidiation is finished, restrictocin would be degraded so that fungus-feeding insects could externally or internally carry spores to new locations for spreading the fungus. Following the idea of a protective function of ribotoxins during maturation of conidia, there are some studies on the different feeding behavior of the fungivorous beetle

Carpophilus freemani upon restrictocin production (Brandhorst et al. 1996). Feeding of *C. freemani* adults on *A. restrictus* decreased during conidia formation, something that was not observed when they fed on *A. nidulans* at the same developmental stage. This deterrence of insect feeding could be related with an increase in restrictocin production by *A. restrictus* and not by *A. nidulans*. Heterologous expression of restrictocin in *A. nidulans* also inhibited insect feeding by *C. freemani* beetles (Brandhorst et al. 2001).

As mentioned before, the production of ribotoxins is not restricted to *Aspergillus* (Liu et al. 1995, Liu et al. 1996, Martínez-Ruiz et al. 1999a, Martínez-Ruiz et al. 1999b, Varga and Samson 2008). The genus *Hirsutella* includes over 50 fungal species that infect many species of invertebrates. The entomopathogen fungus *H. thompsonii* also produces a ribotoxin. This fungus is known to cause natural epizootics in populations of eriophyoid mites. Under *in vivo* conditions, the conidia of *H. thompsonii* attach to the cuticle of the host, germinate and penetrate through it. The hyphae then grow inside the insect and reproduce asexually, contacting new hosts to begin a new cycle of infection (Peng et al. 2002). Studies of the toxicity and pathology of crude filtrates of this fungus during the 1990's revealed a high specificity for the subclass *Acari*, although it was also toxic for other arthropods like moth, flies and mosquito larvae (Vey et al. 1993). The role of toxins in the pathogenesis of the insect infection by *H. thompsonii* has been investigated as well. It was known that other entomopathogen fungi like *Metarhizium anisopliae* or *Bauveria bassiana* produce cyclic peptides that are toxic to insect (de Bekker et al. 2013). These peptides induced immediate paralysis and quick mortality when injected into *Galleria mellonella* larvae. Symptoms upon injection of *H. thompsonii* culture filtrates were different and rather slower, but still lethal for *G. mellonella* larvae. Several investigations led to the identification of Hirsutellin A, a protein produced by *H. thompsonii var thompsonii* with insecticidal activity against *G. mellonella* and *Aedes aegypti* larvae (Liu et al. 1995, Mazet and Vey 1995). This toxin was purified and characterized, showing ribosomal inhibiting activity (Liu et al. 1996) resembling that of ribotoxins. Analysis of 162 strains of *H. thompsonii* revealed the presence of the HtA gene in 100 strains but the expression of HtA was poorly correlated with the mortality rates induced by the broth filtrates in *G. mellonella*, suggesting that there could be additional toxic factors involved in the insecticidal activity of the fungus (Maimala et al. 2002). There are no studies on the exact location of HtA in the fungus, like those performed with *A. restrictus.* Therefore the production of this toxin cannot be linked to a developmental stage yet. Soon after its discovery, HtA was related to ribotoxins due to its ability to specifically cleave rRNA on *Spodoptera frugiperda* cells (Sf9), inhibiting cell growth (Liu et al. 1995). HtA was then subjected to a fine structural and functional characterization, demonstrating that despite its lower size and sequence identity, it was a ribotoxin (Herrero-Galán et al. 2008, Viegas et al. 2009, Herrero-Galán et al. 2012a, Herrero-Galán et al. 2012b).

In fact, characterization of HtA opened the possibility that ribotoxins in general may act as natural insecticides. This idea is also supported by the fact that *Aspergillus*, the main ribotoxin producer known so far, shares feeding niche, such as the farm-stored grain, with several insects, and as a way to compete for survival they could have developed weapons like ribotoxins. Purified restrictocin included in the insects' diet killed *C. freemani* and *S. frugiperda* larvae, and deterred feeding of adult *C. freemani* (Brandhorst et al. 1996). A detailed comparison of the activity of HtA and α-sarcin proved that the latter is nearly as toxic as HtA against insect larvae, insect cell cultures and insect ribosomes (Olombrada et al. 2013). Biochemical characterization of the activity of ribotoxins against insect cells showed how HtA and α-sarcin cause protein synthesis inhibition and the release of the characteristic α-fragment resulting from the rRNA cleavage by ribotoxins (Olombrada et al. 2013), just as it has been previously shown with ribosomes of different origin (Lacadena et al. 2007, Herrero-Galán et al. 2008, García-Ortega et al. 2010). In the conditions assayed, HtA is significantly more active than α-sarcin against isolated insect ribosomes, suggesting that the passage across the cell membrane might be the rate-limiting step for their cytotoxic activity. Of course, in a natural environment additional factors may influence their efficiency, such as their natural biosynthesis and extracellular export, stability in a particular environment or accessibility to their target.

The fact that ribotoxins can inactivate every ribosome known raises the question of how fungi protect their own ribosomes (Miller and Bodley 1988). Ribotoxins are synthesized as precursors that mature into cellular membrane compartments. Protection of the producing cells must rely on a very efficient recognition of the signal sequences and an adequate compartmentalization before they are secreted to the extracellular medium (Endo et al. 1993a, Endo et al. 1993b, Martínez-Ruiz et al. 1998, Lacadena et al. 2007). In fact, when the leader sequence was altered or changed by a different one, heterologous expression of restrictocin in *A. nidulans* showed reduced transcript levels and enhanced cellular lysis, and the ribotoxin was not always localized in the conidiophores of the fungus (Brandhorst and Kenealy 1995), suggesting that the native leader sequence of restrictocin is highly efficient in its role of protecting fungi during secretion. The restrictocin gene in *A. restrictus* has an intron at the beginning of its sequence (Lamy and Davies 1991). Recently, a study has proven that introns can be used to attenuate toxicity of barnase for its use in suicide gene therapy (Chen 2012), so perhaps the existence of an intron in restrictocin is an additional example of the strategies used by these fungi to attenuate ribotoxins' toxicity against their own ribosomes during its synthesis and secretion to the extracellular medium.

The potential biotechnological uses of fungal ribotoxins

I. IMMUNOTOXINS

Over the past two decades fungal ribotoxins have been deeply studied and their remarkable specific ribonucleolytic action is now relatively well understood in molecular terms (Lacadena et al. 2007). Even though the role of these toxins in nature is not yet quite well determined, they already have been tested for their use as therapeutic agents. In fact, they were first discovered as antitumoral agents, although further studies revealed an unspecific cytotoxicity against non-tumoral cells (Roga et al. 1971). Later on, the interest of ribotoxins revived since they could be used as components of immunotoxins used in antitumor therapies. Immunotoxins are usually chimeric molecules composed of a specific antibody fragment responsible for targeting to a specific cell type linked to a toxin moiety which promotes cell death (Fig. 4) (Reiter and Pastan 1998, Kreitman 2001). To overcome the great size of the full-length antibody that could hinder its penetration through solid tumors, new strategies have been developed that use only the antibody variable domains. Regarding the toxic moiety, several toxins such as ricin, *Pseudomonas* exotoxin A or diphtheria toxin have been used (Kreitman 2006). Despite being highly effective, in many cases they showed undesirable side effects.

Fungal ribotoxins show several features that make them appropriate candidates for the construction of immunotoxins: their small size, high thermostability, poor immunogenicity, resistance to proteases and, most importantly, their high efficiency inactivating ribosomes. Accordingly, several colon cancer-specific immunotoxins containing ribotoxins (α-sarcin), or even other fungal RNases (RNase T1) as their toxic component, have been designed and characterized (Carreras-Sangrà et al. 2012, Tomé-Amat et al. 2012). HtA has also been used to create an immunotoxin, using an engineered variant that is unable to cross membranes but still retains the ribonucleolytic activity (Tome-Amat et al. 2015a). This variant seems to be innocuous to cells that do not contain the specific antigen. Therefore, it should be safer, with reduced unwanted side effects during cancer therapy.

The toxicity of an immunotoxin depends on many molecular aspects, such as the antigen binding affinity, internalization rate, intracellular processing, toxin release and intrinsic toxicity. Therefore, the intracellular trafficking of the toxic moiety can modulate the cytotoxic effects of the immunoconjugate. Accordingly, the intracellular trafficking of ribotoxin-based immunotoxins has also been studied, showing how they are internalized via early endosomes and then follow different pathways depending on the toxin used until they reach the cytosol (Tome-Amat et al. 2015a, Tome-Amat et al. 2015b). Finally, a step further in the therapeutic use of ribotoxin-based immunotoxins has been done showing *in vivo* the efficiency of the α-sarcin based immunotoxin IMTXA33αs, which inhibits tumor growth as well as angiogenesis in *nude* mice

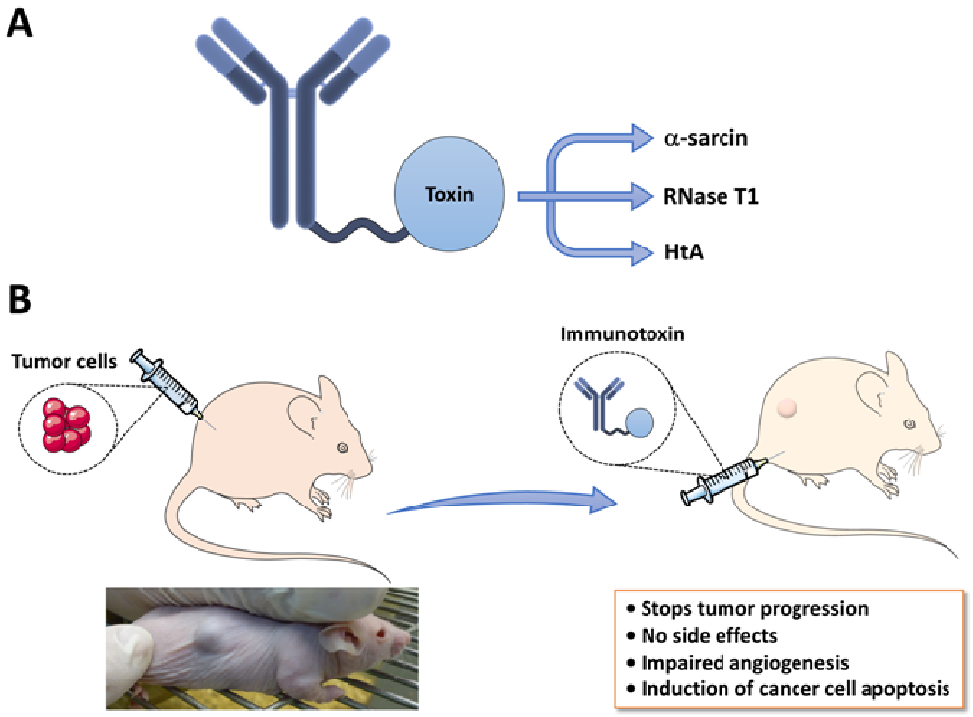

Figure 4.- Ribotoxins as immunotherapeutic agents. (A) General structure of an immunotoxin, composed of a specific antibody fragment responsible for targeting to a specific cell type linked to a toxin moiety, ribotoxins or non-toxic ribonucleases like RNase T1, which promotes cell death. (B) IMTXA33αs, an immunotoxin that contains α-sarcin as the toxic domain, has been tested *in vivo*, showing that it can inhibit tumor growth as well as angiogenesis in nude mice harboring colon cancer xenografts.

harboring colon cancer xenografts (Fig. 4) (Tome-Amat et al. 2015c). A result that can be considered as the proof of concept that immunotoxins based on ribotoxins can constitute a unique therapeutic tool against different tumoral pathologies.

II. ROLE IN ALLERGY AND ASPERGILLUS INFECTION

Aspergillus spp. can also be human opportunistic pathogens that cause respiratory diseases like asthma, ABPA (allergic bronchopulmonary aspergillosis), aspergilloma and severe infections especially in immunocompromised people. *A. fumigatus* is the ethiological agent in 80% of *Aspergillus*-related diseases. The ribotoxin Aspf1 is one of the main allergens of this fungus (*Arruda et al. 1992*). Although Aspf1 is not detected during the initial steps of infection but after cell mass accumulation, it seems to play a role in allergic-like pathogenic processes and some regions have been found to be significantly allergenic (Kurup et al. 1998, García-Ortega et al. 2005). Diagnose and immunotherapy against *A. fumigatus* allergy is frequently based on fungal extracts, but these are usually highly complex mixtures, with hundreds of different components very difficult to standardize, not to mention that there is a high risk of anaphylactic side effects. Improvement of diagnosis and therapeutics has been

focused in the recombinant production of allergens. Since the recombinant Aspf1 still maintains its cytotoxic activity, alternative hypoallergenic ribotoxin molecules have been searched for, produced and characterized, showing that the N-terminal β-hairpin is involved in at least one allergenic epitope (García-Ortega et al. 2005). These non-cytotoxic mutant variants of Aspf1 and α-sarcin might be suitable for their use in immunomodulating therapies for *Aspergillus* hypersensitivity and its diagnosis. Indeed, they have been produced in *Lactococcus lactis*, a GRAS (generally regarded as safe) microorganism that could be used in immunotherapeutic protocols for Aspf1-related diseases as a vaccination vehicle (Álvarez-García et al. 2008). Furthermore, it has been established a mouse model of *A. fumigatus* sensitization that could be suitable for preclinical testing of recombinant allergens and their derivatives (Álvarez-García et al. 2010).

III. RIBOTOXINS AS PEST CONTROL AGENTS

The likely biological role of ribotoxins as insecticides could be used in the design and development of new biopesticides. The world population increases exponentially and it is estimated that by 2050 it will reach 9.5 billion people. This rapid increase of population requires an equal increase in food resources. Pest diseases caused by insects, nematodes, virus and bacteria, as well as competition by weeds, cause up to 40% of looses in agriculture production around the world every year. For decades, pest control has relied on the use of chemical insecticides, such as the use of DDT in the 1940s. They were generally highly potent, fast acting, cheap to produce and easy to deliver. Still, the poor species specificity, its toxicity and rising of pest resistance forced many countries to ban DDT and other chemical pesticides in the 1970s. The problem of new pesticide resistance has continued to grow over the years with the use of new chemical pesticides. By 1992 more than 500 species of mites and insects had developed resistance. Moreover, the cost of discovering, developing and registering new synthetic pesticides is so high that a new interest in alternative biological control methods has risen. Biopesticides have become an important component of environmentally friendly pest management. However, its use has not yet reached its potential, constituting only 2-3% of the insecticidal market (Glare et al. 2012). The term biopesticides includes those pest control techniques that use microbial organisms (bacteria, fungi, viruses and nematodes) and/or secondary metabolites from microorganisms like peptides and proteins. It even comprises genetically modified crops that improve its resistance to insect, fungal, viral or herbicide damage (Carlini and Grossi-de-Sa 2002, Coca et al. 2004). To be considered for its use in pest control, biopesticides must meet several criteria: a) be cheap to produce, b) have broad specificity, c) have a low toxicity in non-target organisms, d) be easy to formulate and deliver, e) remain in the environment enough time to be effective, but not too long to

induce resistance, and f) be publicly perceived as innocuous (Whetstone and Hammock 2007).

Entomopathogen fungi have been used effectively as biological control agents to manage crop diseases, some of them being commercialized, like *Beauveria bassiana,* in the control of numerous insects and flies, or *Metarhizium anisopliae*, recently studied against the mosquito *Anopheles gambiae* (Shaw et al. 2002, Whetstone and Hammock 2007, Kim et al. 2014). *H. thompsonii*, the HtA producer, is also a well-known entomopathogen fungus used in the biological control of, for example, the mite *Varroa destructor*, a honey bee ectoparasite (Kanga et al. 2002, Peng et al. 2002, Shaw et al. 2002). This fungus has been shown to be even more effective than *B. bassiana* and *M. anisopliae* in some cases (Rossi-Zalaf and Alves 2006). *H. thompsonii* was first registered in 1981 as a mycoacaricide (Mycar) for the control of the citrus mite (McCoy and Couch 1982) but formulation problems, lack of field persistence and poor product stability in storage and transport resulted in the loss of this commercial product late in the 1980s. Attempts to overcome these obstacles have been made, evaluating the use of new formulations that improve the delivery, shelf life and field efficacy (Sreerama Kumar and Singh 2008).

Genetic manipulation has also been used to increase virulence of the entomopathogen fungi in pest control (St Leger and Wang 2010). The first successful demonstration of an increased virulence was the overexpression in the fungus *M. anisopliae* of a protease capable of degrading the insect cuticle. This technique has also been used to expand the range of infected species or increase stress resistance. However, no genetically modified (GM) microorganisms are currently commercialized as biopesticides, mostly due to the strict regulations and negative public opinions regarding GM organisms (Glare et al. 2012).

A number of successful biopesticides are based on using compounds produced by the microbes rather than provoking the infection by the whole organism (Glare et al. 2012). Therefore, understanding the mechanism of infection of these fungi as well as the virulence factors involved might help in the design of new formulas for pest control. In this sense, the isolation and characterization of fungal ribotoxins and their activity against insects opens the possibility of using them as bioinsecticides, independently or in formulas where other compounds could be combined with the active agent (Olombrada et al. 2013, Olombrada et al. 2014a). The potential toxicity of ribotoxins against vertebrates could be overcome by the design of new variants such as some HtA Trp-residues mutants (Herrero-Galán et al. 2012b, Tome-Amat et al. 2015a). These proteins retain the exquisite ribonucleolytic activity against the ribosome but they are unable to enter mammalian cells and therefore they also are much less cytotoxic.

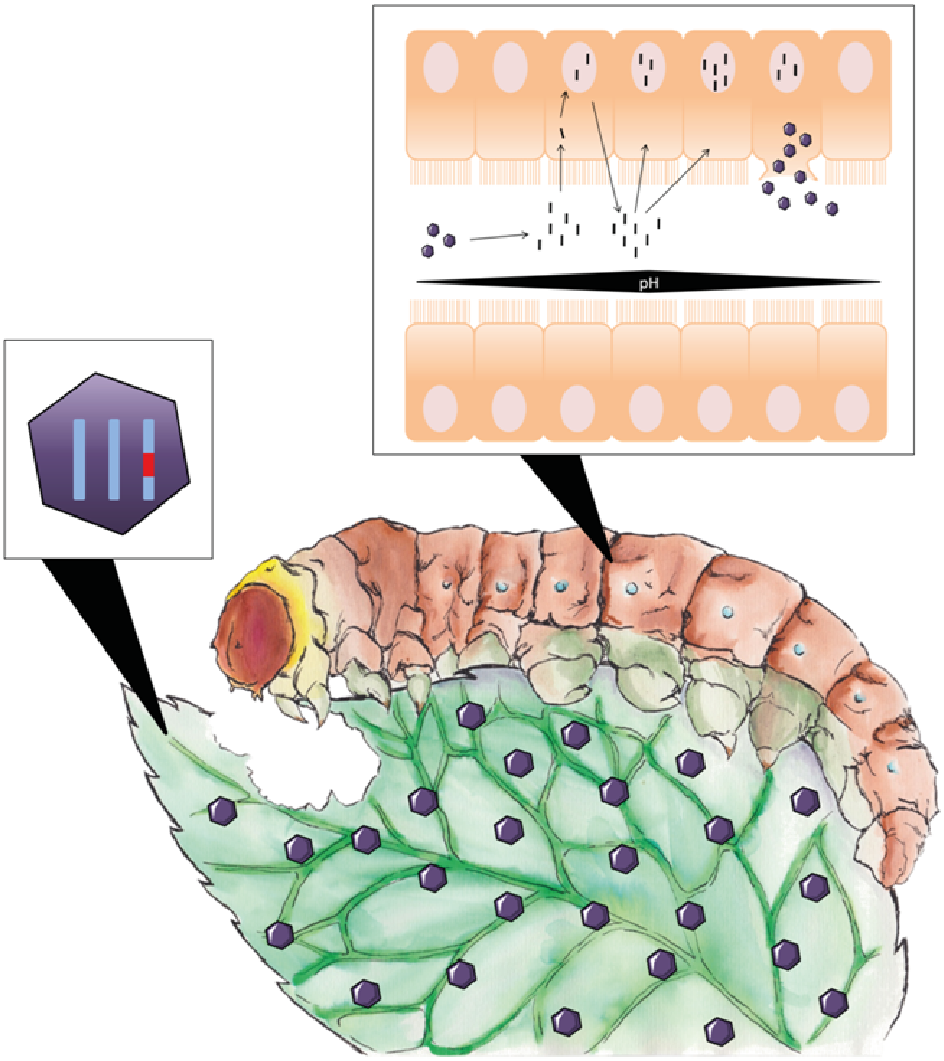

Figure 5.- Baculovirus-based biopesticides. Modified baculoviruses containing genes for insect-specific toxins, hormones or other enzymes are sprayed on the plant foliage and then ingested by the caterpillar or adult insect. Polyhedra get solubilized in the insect's midgut due to an increase of the pH in this region, virions are released and start replicating within the nuclei of epithelial cells, producing more virions which are released in a budded form (early infection) or occluded in polyhedra (late infection).

Due to its intrinsic insecticidal activities, insect pathogenic viruses like baculoviruses represent another strategy for biocontrol (Fig. 5). Natural baculoviruses have been used as effective biopesticides, although genetically engineered and recombinantly produced versions seem to be an even better alternative (Cory et al. 1994, Inceoglu et al. 2001), as they can accelerate the killing speed and do not confer any ecological advantage over the wild-type species. Baculoviruses have the additional advantage of being an easy and stable formula with high specificity for the host. One example with a protein related to fungal pathogenesis is the design of an improved baculovirus that expresses a protease from *A. fumigatus* (Gramkow et al. 2010) that is able to induce death in *Spodoptera frugiperda* larvae faster than the wild-type virus. These results encourage the development of similar tools using ribotoxins. Baculovirus

engineered to produce fungal ribotoxins would be insect-selective, minimizing side toxic effects against other organisms. Furthermore, the extensive knowledge of ribotoxins accumulated over the years may be useful in the design and production of more effective insecticidal toxins. The only concern regarding the use of baculoviruses in pest control is the public opinion about the introduction of GM viruses. However, new environmentally friendly baculoviruses are already being produced, consisting in viruses becoming less active during serial passaging and therefore they become spontaneously inactive after some time (Shim et al. 2013).

IV. THE STUDY OF RIBOSOME-RELATED DISEASES. RIBOSOME BIOGENESIS AS AN ADDITIONAL TARGET OF RIBOTOXINS.

The ribosomes are one of the finest constructed and regulated molecular machines in the cell. They are essential for life, producing all the proteins required for cell growth. In humans, whenever the ribosomal components, or the molecules involved in assuring their correct function and structure, are altered, a heterogeneous number of diseases, named ribosomopathies, appear. These diseases have in common a ribosomal dysfunction, but differ significantly in mechanism, clinical symptoms and potential treatments (Table 1). Some disorders, such as Diamond-Blackfan anemia (DBA), have been linked to mutations in ribosomal proteins, whereas others like the Schwachman-Diamond syndrome (SDS) or the Treacher Collins syndrome (TCS) are directly related to defective ribosome biogenesis (Narla and Ebert 2010, Nakhoul et al. 2014, Ruggero and Shimamura 2014, Warner 2015). Clinical features of these ribosomopathies normally include bone marrow failure, developmental abnormalities and an elevated risk of cancer, although symptoms are diverse, even among patients with the same disease (Narla and Ebert 2010). There are many unanswered questions regarding ribosomopathies. Among them, it is quite striking how, being the ribosome such a conserved machinery among all cells, mutations in different ribosomal proteins can lead to such diversity of phenotypes and how sometimes these mutations affect only specific tissues or cell types. Several possible explanations for this tissue specificity could be: i) division rate is different among cells, so cells that proliferate fast are more affected by defective ribosome biogenesis than those that proliferate slowly; ii) the composition of ribosomes might be different among cell types, with variations in the expression levels of some ribosomal proteins or ribosome-associated factors that in the end can affect the mRNAs being translated (McCann and Baserga 2013, Nakhoul et al. 2014). The elucidation of the basis of these ribosome-related diseases requires a deeper knowledge of the ribosome, especially in the field of its biogenesis, which seems to be altered in a relatively high number of ribosomopathies. *Saccharomyces cerevisiae* has been an important model organism for the study of this process and its relation with not very well known diseases (Menne et al. 2007a, Woolford and Baserga 2013).

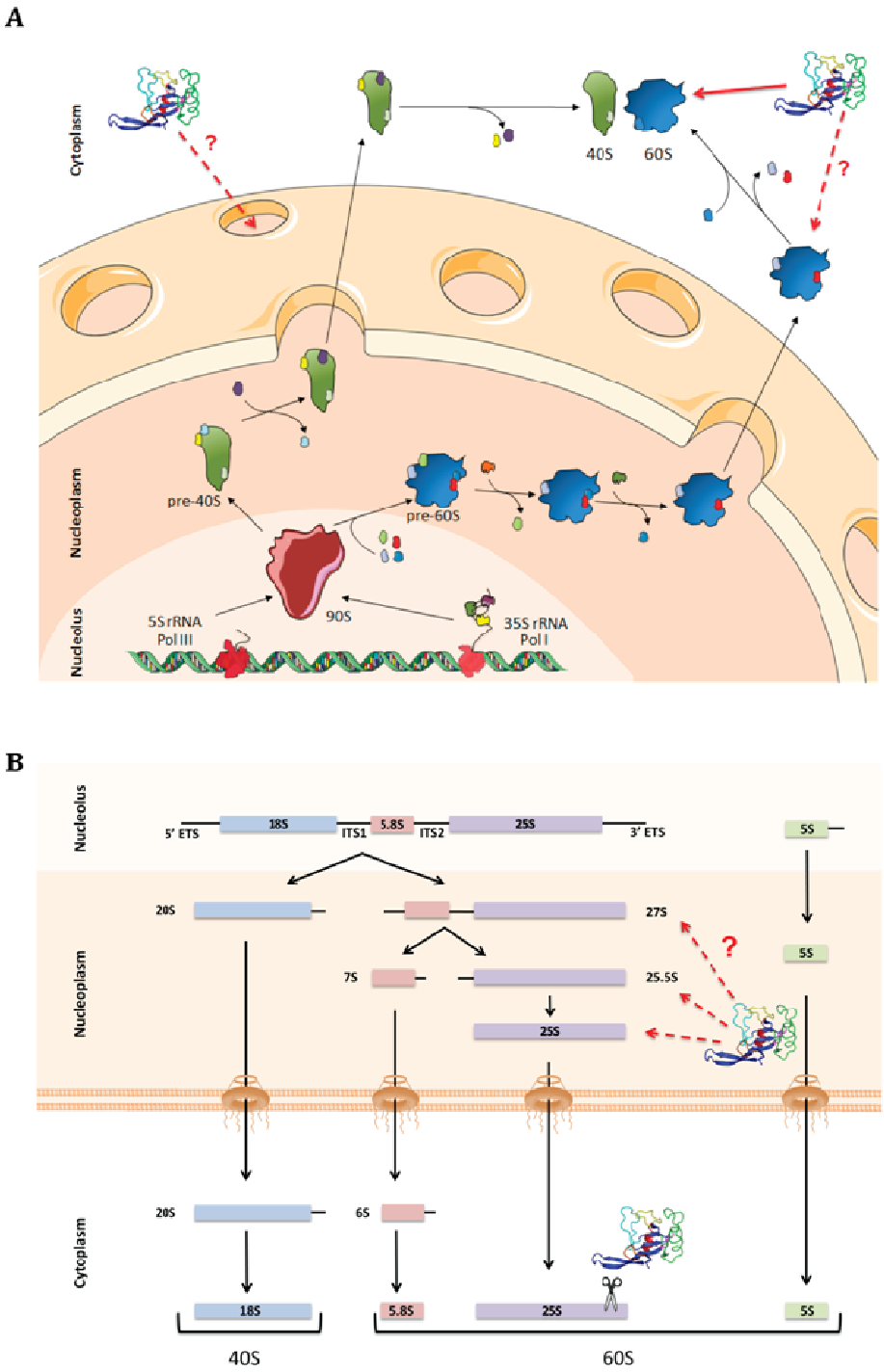

Figure 6.- A) Schematic representation of ribosome maturation in yeast, which starts with transcription of 35S and 5S pre-rRNAs. These rRNAs associate to ribosomal proteins and other factors building the 90S particle. After cleavage of 35S pre-rRNA, the 90S particle separates into pre-60S (blue) and pre-40S subunits (green), which from that point undergo independent maturation through the nucleoplasm and the NPCs. Once in the cytoplasm, final maturation yields translational competent ribosomal particles. Ribotoxins target mature 60S subunits, but pre-60S subunits might also be affected by their toxicity. B) pre-rRNA processing pathway (simplified from Gerhardy et al. 2014). The 35S pre-rRNA common precursor is cleaved yielding 20S pre-rRNA, which is processed to 18S rRNA in the cytoplasm, and 27S pre-rRNA, which is processed to mature 25S rRNA and 5.8S rRNA. The 5S pre-rRNA is processed early in the nucleus. Ribotoxins may affect this processing pathway by cleaving the SRL of mature and pre-rRNAs.

Ribosome biogenesis is a complex cellular process where the large and small ribosomal subunits are assembled. In eukaryotes, this process starts in the nucleolus. Then, both subunits undergo maturation through the nucleoplasm and the cytoplasm (Fig. 6). More than 300 factors, including proteins and RNAs are involved in the production of a eukaryotic ribosome in the yeast *S. cerevisiae*. In humans, this number is even higher, in accordance to the bigger size and complexity of human ribosomes (Lafontaine 2015, Warner 2015). This complex maturation process can be summarized by first describing how 5S and 35S pre-rRNA in yeast are initially transcribed in the nucleolus by RNA polymerases I and III, acquiring post-transcriptional modifications and associating with ribosomal proteins as well as other *trans*-acting factors to form the 90S particle. This particle is then cleaved, releasing pre-40S and pre-60S particles that undergo independent maturation pathways. Pre-40S are rapidly exported to the cytoplasm with relatively few compositional changes, and once in the cytoplasm the main remaining step is the processing of 20S pre-rRNA to yield mature 18S rRNA. Maturation of the pre-60S particle is much more complex and involves several changes of composition when still within the nucleoplasm. Once in the cytoplasm, only a few factors remain attached to the pre-60S particle. A sequential pathway takes place where these factors are released from the pre-60S particles. Major events include the release of factors from the exit tunnel, the assembly of the stalk structure (ribosomal proteins P0, P1 and P2), and the release of the anti-association factor Tif6 (eIF6 in humans), a factor that prevents from premature association of both subunits, by Sdo1 and the GTPase Efl1 (Menne et al. 2007a, Kemmler et al. 2009, Lo et al. 2009, Panse and Johnson 2010, Woolford and Baserga 2013, Gerhardy et al. 2014). Mutations in the human ortholog of Sdo1, SBDS, are responsible for most of the cases of the Shwachman-Diamond syndrome (Brina et al. 2014, Ruggero and Shimamura 2014).

Thanks to powerful techniques like yeast genetics, as well as tandem affinity purification combined with mass spectrometry, the knowledge of ribosome biogenesis has significantly increased over the past 20 years. However, many questions regarding this complex event are still unanswered. Fungal ribotoxins may shed some light in this sense. They might be appropriate tools to explore the maturation pathway of pre-60S particles, especially in the cytoplasm, since their target, the SRL, is localized in a region essential during ribosome maturation. From the ribotoxins' point of view, it would be interesting to see whether these toxins can access the nucleus, and if they do, whether the SRL of pre-60S particles can be cleaved. From the ribosome biogenesis side, understanding how ribotoxins affect mature and immature ribosomes may give some information about pre-rRNA processing and folding, and the function and localization of some trans-acting factors. Eventually, these studies may contribute to a better understanding of pathologies like Shwachman-Diamond syndrome and other ribosomopathies.

Concluding remarks

Over the past decades, the structure and function of fungal ribotoxins have been studied in deep detailed. Today, the accumulated knowledge of this family of toxins has allowed their successful use in some downstream applications, like the design of new specific immunotoxins against colon cancer or the establishment of mouse models for the study of Aspergillosis. The discovery of HtA has opened up the possibility that, in nature, ribotoxins have a major role as defensive toxins with insecticidal activity. Their high specificity against insect cells and larvae make them good candidates for the design and development of new biopesticides. Furthermore, the accumulated knowledge on their structure and function could be also used in the field of ribosome biogenesis, which in the end could contribute to a better understanding of ribosomopathies. In conclusion, even though there is still so much to be done, ribotoxins have great potential to be used as biotechnological tools in many different applications.

Acknowledgements

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Objetivos/Objectives

En esta Tesis Doctoral, se han analizado posibles aplicaciones biotecnológicas de las ribotoxinas fúngicas, centrándose en los siguientes objetivos:

-Estudio de las relaciones estructura-función:

-Análisis e identificación del papel que juegan en la actividad citotóxica residuos de los bucles 2 y 3 de la ribotoxina α-sarcina.

-Estudio del papel de la horquilla β amino-terminal de HtA ya que este elemento estructural es de menor longitud en ésta que en otras ribotoxinas. Análisis de varios mutantes puntuales de su bucle 5, que pudieran estar compensando la falta de cargas y la longitud de la mencionada horquilla.

-Identificación y caracterización de nuevas proteínas de hongos entomopatógenos susceptibles de formar parte de la familia de las ribotoxinas fúngicas. En concreto se ha abordado la producción y caracterización de una nueva ribotoxina, semejante a HtA, y producida por el hongo *Metarhizium anisopliae*.

-Análisis comparativo de la función insecticida de las ribotoxinas fúngicas más representativas, y evaluación de su potencial uso en el control de plagas, como alternativa a los pesticidas tradicionales.

-Estudio de la interacción de las ribotoxinas fúngicas con el ribosoma eucariota, utilizando como modelo la levadura *Saccharomyces cerevisiae*:

-Análisis de la participación del tallo ribosómico eucariota en el mecanismo de interacción de la ribotoxina α-sarcina con el ribosoma.

-Determinación de la actividad de las ribotoxinas fúngicas sobre el proceso de biogénesis del ribosoma eucariota.

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In this Thesis, several possible biotechnological applications of fungal ribotoxins have been studied, focusing on the following objectives:

- Structure-function relationships:

    - Analysis and identification of the role played by several residues of loops 2 and 3 of ribotoxin α-sarcin in its toxic activity.

    - Study of the role of the N-terminal β hairpin of HtA, given its smaller size when compared with other ribotoxins. Analysis of several point mutations in loop 5 that could be playing the role assigned to the N-terminal β-hairpin Lys residues in α-sarcin.

    - Identification and characterization of new proteins that could be part of the family of fungal ribotoxins. Specifically, we aim to produce and characterize a new ribotoxin of similar sequence to HtA found in the genome of the entomopathogen fungus *Metarhizium anisopliae.*

- Comparative analysis of the insecticidal activity of the most representative fungal ribotoxins, evaluating their potential use in pest control as an alternative to classical pesticides.

- Study of the interaction between fungal ribotoxins and the eukaryotic ribosome, using *Saccharomyces cerevisiae* as the model organism.

    - Analysis of the involvement of the eukaryotic ribosomal stalk in the mechanism of interaction of α-sarcin with the ribosome.

    - Study of the influence of the activity of fungal ribotoxins on the eukaryotic ribosome biogenesis pathway.

Resultados/Results

Resultados A: RELACIONES ESTRUCTURA-FUNCIÓN EN LAS RIBOTOXINAS

A1. Participación de los bucles 2 y 3 de la α-sarcina en su actividad ribotóxica.

Las ribotoxinas fúngicas son una familia de RNasas inactivantes del ribosoma extraordinariamente específicas que ejercen su acción sobre la secuencia conservada de rRNA conocida como lazo sarcina/ricina (SRL). La ribotoxina α-sarcina es el miembro de esta familia mejor caracterizado. Su actividad tóxica resulta de la combinación de su actividad RNasa y de su habilidad para cruzar membranas lipídicas. Los bucles 2 y 3 de la α-sarcina parecen estar involucrados en el reconocimiento del SRL y de algunas proteínas del ribosoma, pero también podrían participar en la interacción con las membranas celulares. En este trabajo se han preparado diferentes formas mutantes de la α-sarcina para estudiar la implicación de estas regiones en su acción tóxica. De acuerdo con los resultados obtenidos, los residuos de lisina 111, 112 y 114 del bucle 3 participarían en el reconocimiento del SRL, y uno de éstos , el 114, estaría formando parte de una red de interacciones con la tirosina 48, que es esencial para la catálisis. Los ensayos de interacción con lípidos muestran como este conjunto de lisinas participa también en la interacción con membranas. El bucle 2 parece ser responsable del cambio conformacional que expondría la región de la proteína encargada del establecimiento de interacciones hidrofóbicas con las caras internas de las membranas lipídicas favoreciendo así la entrada de la toxina en las células diana.

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Involvement of loops 2 and 3 of α-sarcin on its ribotoxic activity

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ABSTRACT

Ribotoxins are a family of fungal ribosome-inactivating proteins displaying highly specific ribonucleolytic activity against the sarcin/ricin loop (SRL) of the larger rRNA, with α-sarcin as its best-characterized member. Their toxicity arises from the combination of this activity with their ability to cross cell membranes. The involvement of α-sarcin's loops 2 and 3 in SRL and ribosomal proteins recognition, as well as in the ribotoxin-lipid interactions involving cell penetration, has been suggested some time ago. In the work presented now different mutants have been prepared in order to study the role of these loops in their ribonucleolytic and lipid-interacting properties. The results obtained confirm that loop 3 residues Lys 111, 112, and 114 are key actors of the specific recognition of the SRL. In addition, it is also shown that Lys 114 and Tyr 48 conform a network of interactions which is essential for the catalysis. Lipid-interaction studies show that this Lys-rich region is indeed involved in the phospholipids recognition needed to cross cell membranes. Loop 2 is shown to be responsible for the conformational change which exposes the region establishing hydrophobic interactions with the membrane inner leaflets and eases penetration of ribotoxins target cells.

1. Introduction

Ribotoxins are a family of fungal ribosome-inactivating proteins displaying insecticidal activity (Herrero-Galán et al., 2013, 2008; Olombrada et al., 2013; Olombrada et al. 2014a),with α-sarcin as its best-characterized member (Lacadena et al., 2007). They show a highly specific ribonucleolytic activity against a single phosphodiester bond of the larger rRNA, located at a universally conserved sequence motif known as the sarcin/ricin loop (SRL) (Chan et al., 1983; Endo et al., 1983). Their toxicity arises not only from this exquisite RNase specificity but also from their ability to cross cell membranes. Once inside the cell, they inactivate any known ribosome given the universal conservation of the SRL (Lacadena et al., 2007).

Most known ribotoxins show a high degree of sequence identity, above 85%. The only exception is hirsutellin A (HtA), which is produced and secreted by the invertebrate fungal pathogen *Hirsutella thompsonii* (Mazet and Vey, 1995). This protein stands out because of its significantly smaller size and much lower sequence identity (only 25%; Fig. 1). However, HtA still maintains the characteristic elements of regular secondary structure as well as the nature and geometrical arrangement of most of the residues making the active site (Herrero-Galán et al., 2008; Viegas et al., 2009). On the other hand, HtA displays important differences regarding its non-structured loops. These differences are especially remarkable for the NH_2-terminal β-hairpin and loops 2 and 3 (Fig. 1). Even though, HtA maintains all functional properties of ribotoxins except for a slightly different behavior in assays with phospholipid model vesicles (Herrero-Galán et al., 2008; Yang et al., 2001).

The importance of the long loop 2 (Fig. 1; green loop) in ribotoxins functionality was suggested long time ago. It was proposed to be involved in the recognition of the SRL (Yang et al., 2001) and several ribosomal proteins (García-Mayoral et al., 2005), as well as in the ribotoxin-lipid interactions involving cell penetration (Kao and Davies, 1999; Martínez-del-Pozo et al., 1988; Pérez-Cañadillas et al., 2000; Yang and Moffat, 1996). In α-sarcin, the conformation of this region is stabilized in part by a conserved specific hydrogen bond between Asn54 and Ile69 (Hebert et al., 1998; Pérez-Cañadillas et al., 2002; Siemer et al., 2004), something that is not

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CD, circular dichroism; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; DPX, N,N-p-xilene-bispyridiniumbromide; HtA, Hirsutellin A; SRL, sarcin/ricin loop; TCA, trichloroacetic acid.

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necessary in HtA because loop 2 is much shorter (Fig. 1). It was proposed that α-sarcin interacts with eukaryotic ribosomal protein L9 through the highly basic part of this loop 2, containing Lys residues 61, 64, 70, 73, 81, 84, and 89 (García-Mayoral et al., 2005), a sequence stretch suggested to be also involved in membrane interactions. Interestingly, the HtA shorter loop 2 lacks this important lysine-rich surface. Finally, from structural and functional points of view, the long loop 2 of α-sarcin can be divided into two different segments due to the presence of a disulfide bridge established between Cys 76 and 132. This second segment stretch of loop 2 (residues 77–93) not only contains three of the mentioned Lys residues but also His 82 (Fig. 1), a residue that establishes a well defined cation-π interaction with Trp 51 (De Antonio et al., 2000; Pérez-Cañadillas et al., 1998, 2000). This observation is interesting considering that the protein region containing Trp51 seems to be involved in α-sarcin membrane interactions (De Antonio et al., 2000).

From the point of view of the recognition of their RNA substrate, the most significant features of the SRL structure are a GAGA tetraloop, containing the phosphodiester bond to be cleaved, and a bulged G motif (Correll et al., 1998, 1999). Loop 3 of α-sarcin contains three Lys residues (Lys111, Lys112 and Lys114) (Fig. 1; light blue loop) which seem to be of special importance as they appear to contact this identity bulged-G element of the ribosomal SRL region (Kao and Davies, 1999, 2000; Korennykh et al., 2007; Korennykh et al. 2006; Pérez-Cañadillas et al., 2000; Plantinga et al., 2008, 2011; Yang et al., 2001; Yang and Moffat, 1996). Furthermore, the electrostatic interactions involving these three Lys residues of loop 3 seem to be a major contributing factor for positioning the SRL-like oligo substrate for site-specific cleavage (Plantinga et al., 2008, 2011). Interestingly, only two of these Lys residues appear to be conserved in HtA (Lys 83 and 88) (Fig. 1).

In the work herein presented, different mutant versions of α-sarcin have been produced, isolated and characterized. First, a chimeric version of α-sarcin (ΔSarHtA) has been constructed by replacing the α-sarcin stretch comprising residues 79 to 93 (second segment of loop 2) (Fig. 1; gray loop) by the equivalent and much shorter HtA segment: Ala60-Asp61-Ala62-Ile63 (Fig. 1B, underlined sequences). Second, another mutant has been made in this loop 2 by replacing His82 by a Gln residue (H82Q). Finally, the three singular Lys residues of loop 3 have been also individually mutated to Glu (K111E, K112E, and K114E); involving a charge reversion. The results obtained are discussed in terms of the individual roles of these regions in α-sarcin interactions with ribosomes and biological membranes.

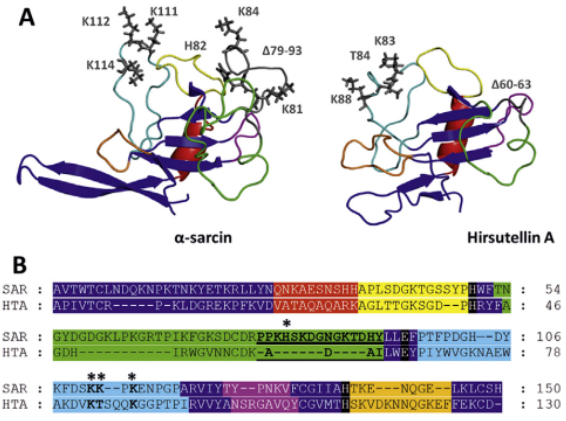

Fig. 1. (A) Diagrams showing the three-dimensional structure of α-sarcin (PDB ID: 1DE3) and HtA (PDB ID: 2KAA). Side chain of the four residues mutated in α-sarcin are shown in gray, as well as the equivalent ones equivalent in HtA, according to the sequence alignment shown in (B). α-Sarcin loop 2 backbone stretch deleted is also shown in gray. (B) Alignment of α-sarcin and HtA amino acid sequences. Residues mutated in α-sarcin are marked with an asterisk. Both the deleted residues in α-sarcin ΔSarHtA, as well as the introduced amino acids corresponding to the equivalent HtA stretch, appear underlined. Color codes: Blue for the NH_2-terminal β-hairpin and the β-strands; red for the α-helix; yellow for loop 1; green for loop 2; light blue for loop 3, magenta for loop 4; orange for loop 5. The diagrams were generated with PyMol software (DeLano, 2008). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Mutant cDNA construction

All materials and reagents were of molecular biology grade. Cloning procedures, PCR-based oligonucleotide site-directed mutagenesis, and bacterial manipulations were carried out as previously described (Álvarez-García et al., 2006; Lacadena et al., 1994; Martínez-Ruiz et al., 2001). Mutagenesis constructions were performed using different sets of complementary mutagenic primers (Table S1). Mutations were confirmed by DNA sequencing at the corresponding Complutense University facility. The plasmid used as template for mutagenesis, containing the cDNA sequence of wild-type α-sarcin, has already been described (Lacadena et al., 1994, 1999).

2.2. Protein production and purification

Escherichia coli BL21 (DE3) cells, previously cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding α-sarcin wild-type or mutant plasmids were used to produce and purify all proteins, as previously described (García-Ortega et al., 2000; Lacadena et al., 1995, 1994, 1999; Siemer et al., 2004). Fungal natural wild-type α-sarcin was produced and isolated as reported before (Martínez-Ruiz et al., 2001). Yields were always in the order of milligram amounts per liter or original culture (Table S2). SDS-PAGE of proteins, Western blots, protein hydrolysis, and amino acid analysis were performed according to standard procedures, also as previously described (Lacadena et al., 1994; Martínez-Ruiz et al., 2001).

2.3. Spectroscopic characterization

Spectroscopic characterization was performed following well-established procedures (Álvarez-García et al., 2009a, 2006; García-Ortega et al., 2001, 2005, 2002; Lacadena et al., 1999; Martínez-Ruiz et al., 2001). Absorbance measurements were carried out on a Beckman DU640 spectrophotometer (Beckman Coulter, Brea, CA, USA) at 200 nm/min scanning speed and room temperature. Amino acid analyses and the corresponding UV-absorbance spectra were also used to calculate their extinction coefficients (Table S2). Circular dichroism spectra were obtained in a Jasco 715 spectropolarimeter (Jasco, Easton, MD, USA), equipped with a thermostated cell holder and a Neslab-111 circulating water bath, at 0.2 nm/s scanning speed. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 220 nm in the 25–80 °C range using a rate of temperature increment of 30 °C per hour. Fluorescence emission spectra were recorded on an SLM Aminco 8000 spectrofluorimeter at 25 °C using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission

beams, respectively, were used. All these experiments were performed in 50 mM sodium phosphate, pH 7.0, containing 0.1 M NaCl.

2.4. Ribonucleolytic activity assays

The ribonucleolytic activity of α-sarcin on rabbit ribosomes was followed by detecting the release of the specific 400-nt α-fragment from the ribosomes of a cell-free rabbit reticulocyte lysate (Promega) as described (Kao et al., 2001). Visualization of this α-fragment was performed by ethidium bromide staining of 2.4% agarose gels after electrophoretic fractionation of the samples using denaturing conditions.

The specific cleavage by ribotoxins of a 35-mer synthetic oligonucleotide mimicking the sequence and structure of the SRL was also analyzed as described before (Kao et al., 2001). The sequence of this oligo was 5′-GGGAAUCCUGCUCAGUACGA-GAGGAACCGCAGGUU-3′, where the cleavage site by α-sarcin appears underlined. Synthesis of this SRL-like RNA oligo was performed as previously described (García-Ortega et al., 2010; Kao et al., 2001). Reaction products were run on a denaturing 19% (w/v) polyacrylamide gel and visualized by ethidium bromide staining.

The absence of contaminating non-specific RNase-like activities in the protein preparations employed was ruled out in all protein batches employed by means of zymogram assays against poly(A) (Kao et al., 2001; Lacadena et al., 1995, 1994, 1999). These assays were also used to evaluate the non-specific residual ribonucleolytic activity of the proteins studied against non-structured substrates.

2.5. Phospholipid vesicle assays

DMPG (1,2-dimyristoyl-sn-glycero-3-phosphoglycerol) was purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, U.S.A.). Vesicles were formed in 15 mM Tris–HCl, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, as previously described (Mancheño et al., 1994; Martínez-Ruiz et al., 2001). Phospholipid concentration was also determined as described (Bartlett, 1959). Analysis of protein binding to vesicles was performed by ultracentrifugation (Alegre-Cebollada et al., 2006; Martínez-Ruiz et al., 2001) using samples prepared at different lipid to protein molar ratios which were incubated at 37 °C for 1 h and then centrifuged at 164000 g for 1 h at 4 °C in a 42.2 Ti Beckman rotor. The amount of protein that did not sediment with the vesicles was determined from the absorbance spectra of the supernatant, and the concentration of ribotoxin bound to the liposomes was then calculated taking into account the initial concentration of protein (10 μM in all cases). Aggregation was monitored as described before (Gasset et al., 1989) by measuring the increase of the absorbance at 360 nm of a suspension of vesicles (30 μM final lipid concentration) after addition of a small aliquot of a freshly prepared solution of protein. Leakage of vesicle aqueous contents was measured by using the 8-aminonaphthalene-1,3,6-trisulfonic acid/N,N-p-xilene-bispyridiniumbromide (ANTS/DPX) assay as previously described (Mancheño et al., 1998). Other experimental details were as previously reported (Gasset et al., 1994, 1995, 1991a, 1991b, 1990; Mancheño et al., 1995; Mancheño et al. 1994; Oñaderra et al., 1993).

2.6. Insect cells culture and toxicity assays

The insect cell line *Spodoptera frugiperda* (Sf9) was cultured as described (Olombrada et al., 2013; Tello et al., 2010) in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker) at 27 °C as indicated by the manufacturer. Protein solutions were prepared in Insect X-press medium and sterilized by ultrafiltration. Protein biosynthesis inhibition assays were made seeding Sf9 cells into 24-well plates at a cell density of 10^5 cells/well and were maintained under standard culture conditions up to 80% confluency (2 days). Then, monolayer cultures were incubated in 0.5 mL of fresh medium with serial dilutions of ribotoxin from 5.0 mM to 0.5 nM final concentrations. Following 18 h of incubation at 27 °C the medium was replaced by culture medium supplemented with 0.5 μCi/well of [^{3}H]-leucine. After 5 h of incubation the medium was removed and cell protein content was precipitated with 5% trichloroacetic acid (TCA) and washed three times with ethanol. The precipitate was dried, dissolved in 200 mL of 0.1 N NaOH, 0.1% SDS and radioactivity was measured in a Beckman LS 3801 liquid scintillation counter. Results are expressed as percentage of incorporated radioactivity relative to samples without protein added.

3. Results

3.1. Protein purification and spectroscopic characterization

Wild-type α-sarcin and the five different mutants produced were purified to homogeneity according to their SDS–PAGE behavior (Fig. S1). Their amino acid composition was consistent with the mutation expected. All of them were also detected by a rabbit anti-α-sarcin serum in Western blot assays (Fig. S1).

Far and near-UV CD spectra of α-sarcin K111E, K112E, and K114E were practically indistinguishable from those ones corresponding to the wild-type protein (Figs. 2 and 3). Only the K114E mutant showed minor changes in the near-UV region. The far and near-UV circular dichroism spectra of ΔSarHtA were different from that corresponding to the wild-type protein (Figs. 2 and 4), but they still corresponded to a structured polypeptide. In fact, the near-UV spectrum revealed the presence of tertiary structure in the mutant variant in spite of the spectral changes observed. The calculated difference spectra wild-type *minus* mutant (Fig. 2) clearly resemble those corresponding to Trp-51 deduced for the W51F mutant variant of the protein (De Antonio et al., 2000). Finally, the α-sarcin H82Q mutant also showed significant variation in both UV wavelengths regions studied (Figs. 2 and 4). These last two spectra were almost coincident to those obtained before for the W51F mutant of the same protein (De Antonio et al., 2000), confirming the already proposed interaction between the side-chains of Trp51 and His82 (De Antonio et al., 2000; Pérez-Cañadillas et al., 1998, 2000).

In accordance with the near-UV CD spectra, the three K111E, K112E, and K114E mutants also showed very similar fluorescence

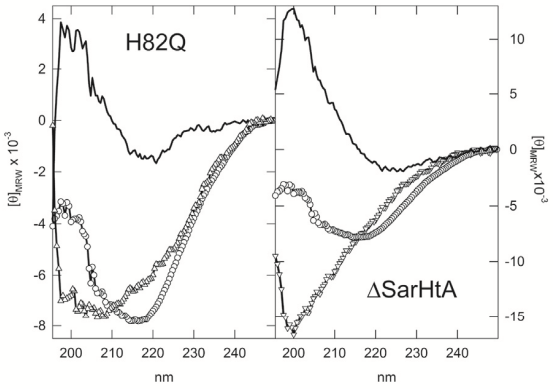

Fig. 2. Far-UV circular dichroism spectra. (*Circles*) Wild-type α-sarcin, (*triangles up*) H82Q variant, and (triangles down) ΔSarHtA. *Black lines*, calculated difference spectra wild-type *minus* mutant, either H82Q or ΔSarHtA. Spectra of K111E, K112E, and K114E are not shown because they were indistinguishable from that obtained for the wild-type protein. Mean residue weight ellipticity (θ_{MRW}) is expressed in units of degree x cm^2 x dmol^{-1}.

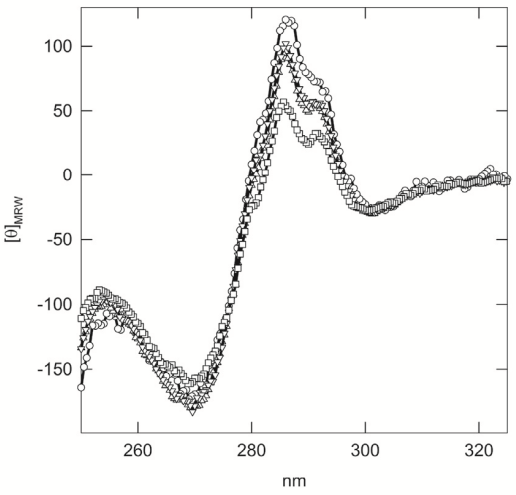

Fig. 3. Near-UV circular dichroism spectra. (*Circles*) Wild-type, (*triangles up*) K111E, (*triangles down*) K112E and (*squares*) K114E mutant variants of α-sarcin. Mean residue weight ellipticity (θ_{MRW}) is expressed in units of degree x cm^2 x $dmol^{-1}$.

emission spectra when compared to the wild-type protein (Table S2, spectra not shown). Fluorescence emission spectra for both ΔSarHtA and H82Q mutants (Fig. 5) showed a remarkable enhancement of their Trp quantum yields (Table S2) and a slight red-shift of about 5 nm. In both cases, this observation is again in agreement with the loss of the His82-Trp51 interaction (De Antonio et al., 2000).

3.2. Ribonucleolytic characterization

Ribotoxins are highly specific RNases against ribosomes. They still retain this specificity when assayed against naked RNA containing the SRL sequence and structure. This is why its ribonucleolytic activity can be studied using a 35mer SRL-like oligomer which lacks any other additional ribosomal structural feature. However, they can also cause extensive, non-specific, progressive digestion of RNA when used at higher concentrations (Endo et al., 1988; Wool, 1996, 1997), including homopolynucleotides (Kao et al., 2001). The loss of specificity of these assays is compensated by the possibility of obtaining useful information about catalysis *per se*, independently of the degree of specific binding of the protein analyzed. Thus, although they are less specific, these assays have contributed significantly to the detailed study of the cleavage

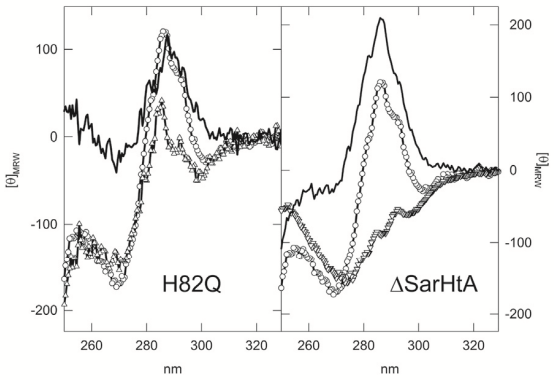

Fig. 4. Near-UV circular dichroism spectra. (*Circles*) Wild-type α-sarcin, H82Q variant (*triangles up*) and ΔSarHtA (triangles down). *Black lines*, calculated difference spectra wild-type *minus* mutant, either H82Q or ΔSarHtA. Mean residue weight ellipticity (θ_{MRW}) is expressed in units of degree x cm^2 x $dmol^{-1}$.

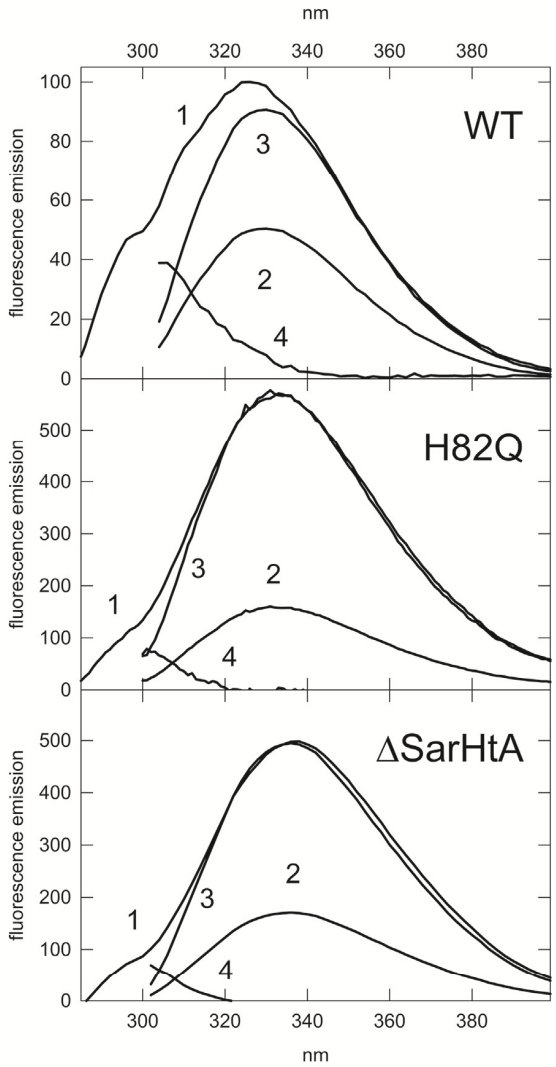

Fig. 5. Fluorescence emission spectra of wild-type α-sarcin and the ΔSarHtA and H82Q mutants. All spectra were recorded at identical protein concentrations. Spectra labeled '1' resulted from excitation at 275 nm and spectra labeled '2' from excitation at 295 nm. These spectra were normalized at wavelengths above 380 nm to obtain spectra '3' (tryptophan contribution). Spectra '4' (tyrosine contribution) were calculated by subtracting spectra '3' from spectra '1'. Fluorescence emission units were arbitrary, and referred to the maximum value of wild-type α-sarcin upon excitation at 275 nm.

mechanism of ribotoxins (Álvarez-García et al., 2009b; García-Ortega et al., 2002; Kao et al., 2001; Lacadena et al., 1995, 1999; Martínez-Ruiz et al., 2001).

The three K111E, K112E, and K114E mutants, as well as the ΔSarHtA variant, were unable to achieve the ribotoxins specific ribonucleolytic cleavage of the ribosomal SRL when assayed in standard conditions against intact ribosomes in a cell-free reticulocyte lysate (Fig. 6A) even at high enzyme concentration (range assayed: 60–200 nM). The α-sarcin H82Q mutant retained about 80% of the wild-type protein ribonucleolytic activity (Fig. 6A). Nearly identical results were also obtained when using a 35-mer SRL-like oligoribonucleotide (Fig. 6B), a less specific substrate. Finally, the ribonucleolytic activity was tested using zymogram assays performed in polyacrylamide gels containing the non-specific substrate homopolynucleotide poly(A) (Fig. 6C). In this case, only the ΔSarHtA mutant did not show detectable activity,

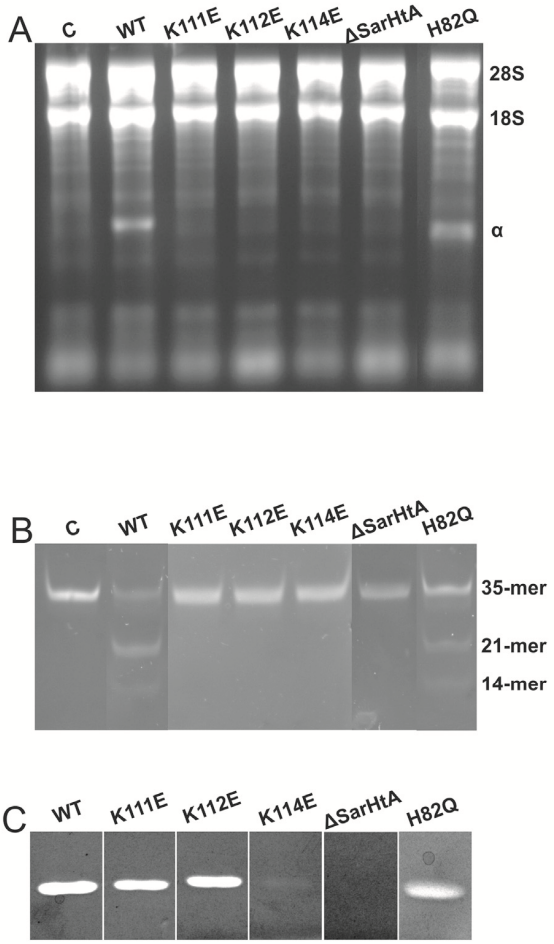

Fig. 6. Ribonucleolytic characterization of wild-type α-sarcin and the mutant proteins studied. (A) Ribosome cleaving activity assay performed using a rabbit cell-free reticulocytes lysate. A control in the absence of enzyme is also shown (c). The highly specific ribonucleolytic activity of the ribotoxins is shown by the release of the 400-nt α-fragment (α) from the 28S rRNA of eukaryotic ribosomes. Positions of bands corresponding to 28S and 18S rRNA are also indicated. (B) Activity assay on a 35-mer oligonucleotide mimicking the SRL. A control in the absence of enzyme is also shown (c). The 21-mer and 14-mer oligonucleotides resulting from the specific cleavage of a single phosphodiester bond are indicated. The intact 35-mer oligo is also shown. (C) Zymogram assay corresponding to the non-specific ribonuclease activity of the proteins. The poly(A)-degrading activity of the proteins produces a colorless region.

while the K111E, K112E and H82Q mutants were as active as the wild-type. Interestingly, the α-sarcin K114E variant was still much less active (Fig. 6C). These zymogram experiments were also employed to discard the presence of trace amounts of non-specific ribonucleolytic contaminants in the samples used.

Overall, this ribonucleolytic characterization suggested that ΔSarHtA had lost completely its ability to behave as an RNase while the three Lys to Glu mutants were devoid of the distinctive and unique specificity of ribotoxins but still maintained non-specific activity against single stranded non-structured RNA stretches. α-Sarcin H82Q still showed a very similar ribonucleolytic behavior as the wild-type protein.

3.3. Interaction with phospholipid vesicles

All protein variants studied bound to DMPG vesicles showing very similar behavior and stoichiometry as the wild-type α-sarcin (Fig. 7A). Only K111E and K114E showed a slightly diminished binding affinity in terms of the amount of protein needed to reach saturation. In accordance with this observation, these two variants were also less effective in aggregating the vesicles (Fig. 7B), suggesting that conserved Lys 111 and Lys 114 residues participate not only in SRL recognition but also in the protein-lipid interactions needed to enter the ribotoxins cellular targets. α-Sarcin mutants

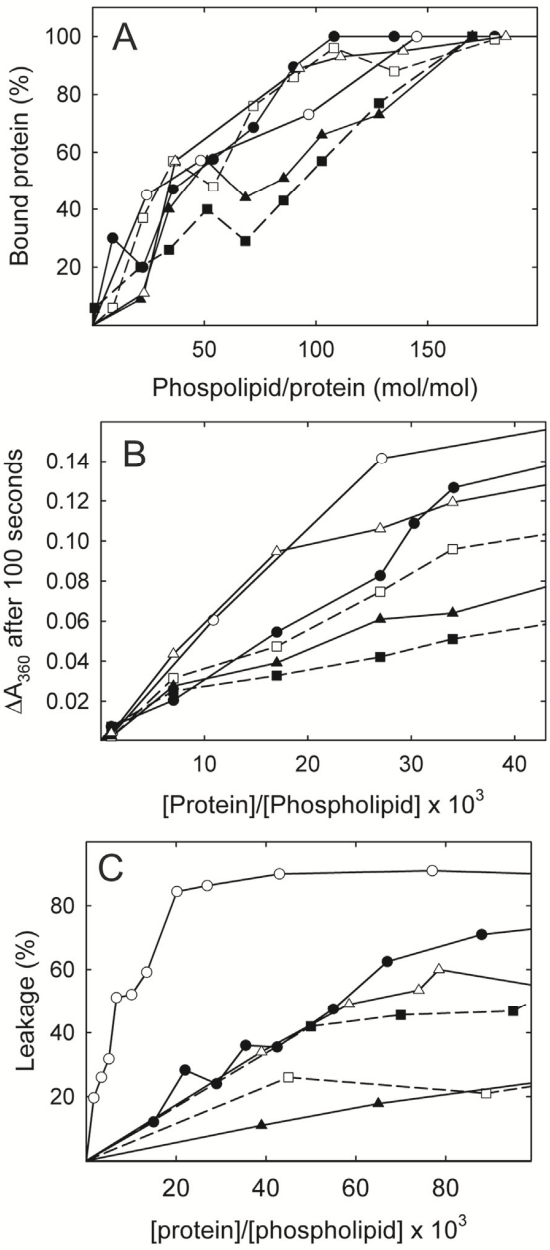

Fig. 7. (A) Binding of wild-type α-sarcin and the different mutants studied to DMPG vesicles. Protein bound to the vesicles (expressed as percentage) was calculated as the difference total protein minus protein remaining in the supernatant after ultracentrifugation, 100% value being the total amount of protein present. (B) DMPG vesicle aggregation induced by wild-type α-sarcin and the different mutants. Aggregation of vesicles was measured as the increase of absorbance at 360 nm, 100 s after the addition of the protein to a vesicle sample (ΔA_{360} nm versus protein/lipid molar ratio). (C) Leakage of intravesicular aqueous contents (relative leakage considering that produced by detergent as 100%) versus protein/lipid molar ratio. Graph symbols correspond to wild-type (black dots), H82Q (white dots), K111E (black squares), K112E (white squares), K114E (black triangles), and ΔSarHtA (white triangles).

H82Q and ΔSarHtA showed the opposite behavior, displaying enhanced aggregation ability.

It is well known that α-sarcin interacts with lipid vesicles containing an abundance of acid phospholipids. This interaction results in vesicle aggregation, intermixing of phospholipids from different vesicles, and leakage of the aqueous contents of its target vesicles. However, HtA does not promote vesicle aggregation, but still perturbed the permeability barrier of the phospholipid bilayers as shown in leakage experiments (Herrero-Galán et al., 2008). Quantitatively, HtA had in fact a higher membrane-permeabilizing ability than α-sarcin (Herrero-Galán et al., 2008). Therefore leakage induced by the present α-sarcin mutants was also studied. As can be observed in Fig. 7C, ΔSarHtA and K111E mutants showed leakage activity almost indistinguishable from that displayed by the wild-type protein, while the other two lysine mutants, K112E and K114E, were less active. The most striking result was however obtained for α-sarcin H82Q. This mutant produced massive release of the phospholipid vesicle aqueous contents at much smaller concentrations than the other proteins, wild-type α-sarcin or HtA (Herrero-Galán et al., 2008) included. This result suggests that the substitution of His 82 by a Gln residue produces a local conformational change which most probably exposes lipid interacting residues which remain buried in the wild-type protein.

3.4. Toxicity against cultured insect cells

It has been recently shown the dramatic effect of wild-type α-sarcin on the inhibition of *in vivo S. frugiperda* cells protein biosynthesis (Olombrada et al., 2013). Therefore, this insect cellular line was chosen to evaluate the toxic effect of the mutants studied when employed against intact cells. As can be seen in Fig. 8, with the only exception of α-sarcin H82Q, all the other mutants were virtually devoid of toxic activity when evaluated in terms of the IC_{50} values needed to inhibit protein biosynthesis. Interestingly, and in good agreement with all the other results showed above, the H82Q mutant showed a toxic behavior which was indistinguishable from that shown by the wild-type ribotoxin.

4. Discussion

4.1. Structural characterization

The spectroscopic characterization of the different α-sarcin protein variants showed that only very minor structural modifications were introduced upon replacing Lys 111, Lys 112, or Lys 114 by a glutamic acid residue. Only K114E showed clear changes in the near-UV CD spectrum related to some aromatic amino acid (Fig. 3), suggesting that switching one positive charge by a negative one may influence the microenvironment of some aromatic residue located in the vicinity of Lys 114 side-chain. In fact, in the wild-type protein structure of α-sarcin the phenol moieties of Tyr 48 and Tyr 106 are within hydrogen bonding distances of the ε amino group of Lys 114 (Fig. 9). The other two mutated Lys residues are oriented towards the solvent (Figs. 1 and 9). Accordingly, the stability of K111E and K112E remained unaltered in terms of their T_m values (Table S2), while it decreased for K114E. Altogether, and taking into account that α-sarcin fluorescence emission spectrum is dominated by Trp 4 (De Antonio et al., 2000), this geometrical arrangement would also explain why the fluorescence emission of the three Lys to Glu variants remained practically unaffected.

The other single residue mutant variant, H82Q, showed unique spectroscopic features. Both far- and near-UV spectra changed substantially (Figs. 2 and 4) and a dramatic Trp emission enhancement was observed (Fig. 5 and Table S2). The CD changes can be easily explained considering that it has been well documented how Trp 51 is not only responsible for most of the optical anisotropy of α-sarcin in the near-UV region but also that it displays a significant contribution in the far-UV (De Antonio et al., 2000). This observation was explained by the cation-π interaction established between the Trp51 side-chain and the ring of His 82 (De Antonio et al., 2000), an interaction which would be absent in the H82Q mutant. Accordingly, the far-UV CD spectrum of this variant (Fig. 2) is practically indistinguishable from that one reported before for the W51F mutant (De Antonio et al., 2000). Regarding the fluorescence emission results of α-sarcin H82Q (Fig. 5), the positively charged His 82 imidazol ring promotes strong quenching on the indole side-chain of Trp 51, the emission of the wild-type protein arising from Trp-4 (De Antonio et al., 2000). Therefore, a large increase in the fluorescence emission of Trp-51 must be observed upon disappearance of the H82-Trp-51 interaction in the H82Q mutant variant, and would also explain why this H82Q mutant shows the lowest T_m value of all the proteins herein studied (Table S2).

A dramatic structural change was introduced in the ΔSarHtA mutant since the α-sarcin loop 2 stretch (PPKHSKDGNGKTDHY)

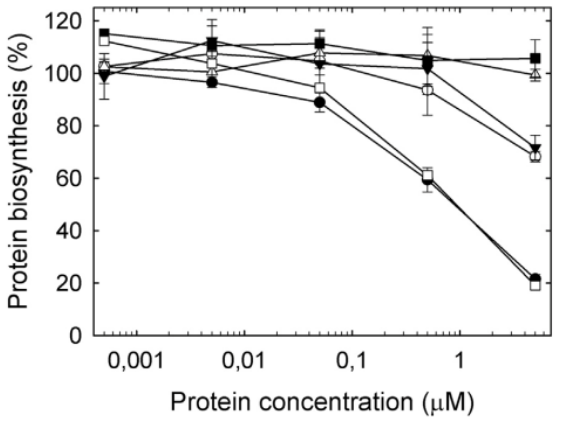

Fig. 8. Protein biosynthesis inhibition in Sf9 insect cells cultured in the presence of different concentrations of wild type α-sarcin (black dots), H82Q (white squares), K111E (black triangles down), K112E (white triangles up), K114E (black squares), and ΔSarHtA (white dots).

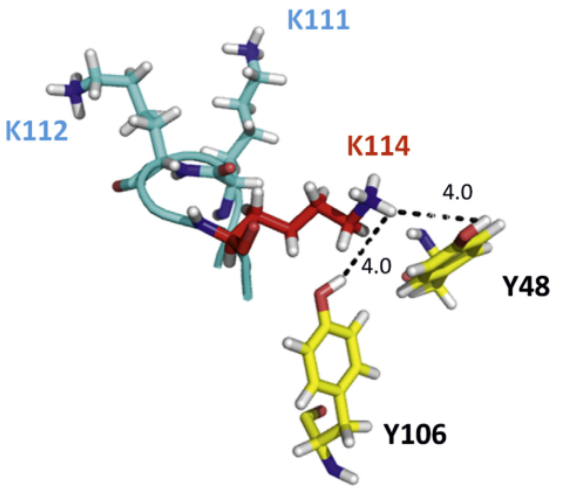

Fig. 9. Diagram showing the spatial distribution of Tyr 48 and 106 (yellow) and Lys 111, 112 (blue) and 114 (red) in wild-type α-sarcin (PDB ID: 1DE3). Diagram was generated using the PyMol software (DeLano, 2008). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was replaced by the sequence ADAI at the equivalent region in HtA. Nevertheless, the mutant protein is still folded, as the CD study revealed, and it seemed to retain most of the folding of the wild-type protein. The existence of a well-defined two-state thermal transition of this protein variant, with a T_m value of only four degrees lower than the natural protein (Table S2), confirmed the adoption of a globular conformation. In addition, the calculated CD spectra WT *minus* ΔSarHtA resemble the Trp51 optical anisotropy contribution (De Antonio et al., 2000). In fact, the CD and fluorescence emission properties of the ΔSarHtA protein variant, where His-82 is absent, are also strongly similar to those of H82Q.

4.2. Ribonucleolytic activity

Only the H82Q mutant retained the unique and highly specific ribonucleolytic activity against ribosomes which is the distinctive feature of fungal ribotoxins (Fig. 6A). This is in good accordance with the previously observed minor changes in the enzymatic activity of W51F α-sarcin (De Antonio et al., 2000). On the contrary, the three lysine residues mutated have been suggested to be important for the specific recognition of the SRL (Dey et al., 2007; Glück and Wool, 2002; Kao and Davies, 1999, 2000; Korennykh et al., 2007; Korennykh et al. 2006; Pérez-Cañadillas et al., 2000; Plantinga et al., 2008, 2011; Yang et al., 2001; Yang and Moffat, 1996). It has been even described that these lysine residues would contribute to the formation of the enzyme:substrate complex (when assayed against a SRL-like oligo), thereby positioning it for site-specific cleavage (Plantinga et al., 2008). An observation which agrees with the fact that the Lys 114 residue has been reported to contact the bulged-G in a sequence specific manner (Glück and Wool, 2002; Yang et al., 2001). Therefore, these results of enzymatic activity would confirm the already proposed key role for these Lys residues in SRL recognition.

However, the most striking result obtained refers to the ability of α-sarcin to cleave rather non-specific substrates such as poly(A). As can be seen in Fig. 6C, replacing the solvent exposed Lys 111 and 112 residues does not affect the ability of this protein to cleave this homopolynucleotide in zymogram assays. This would also agree with the mentioned role of these residues in the specific recognition of the SRL rather than taking part in the catalysis. That is to say, they are key residues for specific binding, but not for catalysis. In fact, it has been described how replacing the mitogillin ribotoxin Lys 111 (the equivalent of α-sarcin Lys 112) by Gln produced a slightly more active mutant than the wild-type protein when assayed against poly(I) (Kao and Davies, 2000). On the other hand, α-sarcin K114E is almost devoid of ribonucleolytic activity, even against the non-specific substrate (Fig. 6C). Taking into account the spatial relationship established between the ε amino group of Lys 114 and the OH of Tyr 48 (Fig. 8), and given that removal of that OH renders a completely inactive version of α-sarcin (Álvarez-García et al., 2006), this interaction would explain why the K114E mutant is also unable to cleave poly(A) and shows how these two amino acids, Lys 114 and Tyr 48, must conform a network of interactions which is essential for the catalysis exerted by this protein.

Finally, the ΔSarHtA mutant was fully inactive against the three type of substrates assayed, suggesting that the second segment of loop 2 affects the geometrical arrangement of the active site.

4.3. Interaction with phospholipid vesicles

The SRL is a universally conserved ribosomal structure but cells are only killed if ribotoxins cross their membranes to gain access to the ribosomes. As no protein receptors have so far been reported for α-sarcin, its toxic specificity has been related to a differential interaction with the negatively charged phospholipids of the membranes (Gasset et al., 1989). Using light-scattering stopped-flow kinetics it was revealed that the initial step of the interaction of α-sarcin with phospholipid vesicles is the formation of vesicle dimers maintained by protein–protein bridges (Mancheño et al., 1994). This initial aggregation is followed by a destabilizing effect of the protein which promotes vesicles fusion. As a final step, and most probably as a consequence of the formation of large unstable lipidic structures, α-sarcin also modifies the permeability of the membranes, causing leakage of the vesicles contents (Gasset et al., 1990).

According to the accepted hypothesis to explain the innate ability of α-sarcin to translocate across a phospholipid membrane (Oñaderra et al., 1993), the protein would be initially adsorbed to the charged polar head groups of the phospholipids and then would penetrate the interface of the bilayer, establishing a hydrophobically driven interaction with the lipid hydrocarbon chains (Gasset et al., 1991a, 1991b). Within this idea, the region comprising a hydrophobic stretch of 24 amino acids (residues 116–139) would be responsible for this hydrophobic component of the interaction (Mancheño et al., 1995, 1998). This sequence is contiguous to the mutated lysine residues of loop 3 (Fig. 1B) and contains His 137, one of the catalytically essential residues of α-sarcin (Lacadena et al., 1995, 1999). Finally, using two different Trp mutants, it was also shown that neither Trp4 nor Trp51 were required for the interaction of α-sarcin with lipid membranes (De Antonio et al., 2000). However, this interaction promoted a large increase in the quantum yield of Trp51, the residue interacting with the indole side-chain of His 82. These results indicated that the region around Trp 51, the first β-strand of the β-sheet, is also located near the hydrophobic core of the bilayer following interaction with the vesicles.

The results presented now reveal no major differences when binding was studied by ultracentrifugation. This approach primarily accounts for the tightly bound protein fraction, most probably that one driven by the hydrophobic component. Consequently, these results could be expected given that the 116-139 sequence has not been modified in any of the mutants herein studied. If we focus on the vesicle aggregation experiments (Fig. 7B), three different types of behavior can be observed. K112E mutant behaves indistinguishable from the wild-type α-sarcin. On the contrary, K111E and K114E show a diminished aggregation ability suggesting that these two Lys residues participate in the electrostatic interactions needed to bring the vesicles into close contact, as had been suggested before (Kao and Davies, 1999; Martínez-del-Pozo et al., 1988; Pérez-Cañadillas et al., 2000; Yang and Moffat, 1996). This proposal would also agree with the results shown in Fig. 6C, where the mutants showing less leakage capacity also involve residues of this Lys-rich region. The other two mutants (H82Q and ΔSarHtA) slightly favored aggregation. Relaxing the protein structure by the absence of the mentioned cation-π interaction involving His-82 would result in exposure of hydrophobic areas promoting an improved membrane destabilizing activity of the polypeptide, easing the interaction with the membrane hydrophobic inner leaflets and the lysis of the vesicles.

4.4. Toxicity against intact cells

The unique and highly specific ribonucleolytic activity of ribotoxins is their trademark feature which explains their extreme toxicity once inside a cell (Endo et al., 1983; Lacadena et al., 2007; Olombrada et al., 2014b). Therefore, and given that only the H82Q mutant retained this highly specific ribonucleolytic activity against ribosomes (Fig. 6A), it is quite straightforward to explain why this mutant was also the only one retaining the α-sarcin ability to

inhibit protein biosynthesis when assayed against intact cells (Fig. 8).

5. Conclusions

The role of α-sarcin's loop 2 in the toxic activity of this protein has been only poorly studied so far. On the other hand, the Lys-rich cluster of loop 3 is well acknowledged as a key element in the specific recognition of the SRL. The results presented now confirm these observations for loop 3 Lys111, Lys112, and Lys114 residues. In addition, they also show the existence of a previously undetected network of interactions involving Lys114 and Tyr48 residues which seems to be essential for the catalysis exerted by α-sarcin. Finally, the Lys mutants studied now involve a change from positive to negative charge which impairs the interaction with the lipid vesicles. Therefore, this charge reversal reveals that this Lys-rich region of loop 3 is involved in the electrostatic phospholipid interactions needed by ribotoxins to cross cell membranes. Regarding loop 2, it is also shown now how this loop seems to be responsible for the conformational change that exposes the region establishing the hydrophobic interactions with the membrane inner leaflets, easing penetration of ribotoxins into their target cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2015.01.007.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2015.01.007.

References

Alegre-Cebollada, J., Rodríguez-Crespo, I., Gavilanes, J.G., Martínez-del-Pozo, A., 2006. Detergent-resistant membranes are platforms for actinoporin pore-forming activity on intact cells. FEBS J. 273, 863–871.

Álvarez-García, E., García-Ortega, L., De los Ríos, V., Gavilanes, J.G., Martínez-del-Pozo, A., 2009a. Influence of key residues on the heterologous extracellular production of fungal ribonuclease U2 in the yeast Pichia pastoris. Protein Expr. Purif. 65, 223–229.

Álvarez-García, E., García-Ortega, L., Verdun, Y., Bruix, M., Martínez-del-Pozo, A., Gavilanes, J.G., 2006. Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of α-sarcin. Biol. Chem. 387, 535–541.

Álvarez-García, E., Martínez-del-Pozo, A., Gavilanes, J.G., 2009b. Role of the basic character of α-sarcin's NH_2-terminal β-hairpin in ribosome recognition and phospholipid interaction. Arch. Biochem. Biophys. 481, 37–44.

Bartlett, G.R., 1959. Colorimetric assay methods for free and phosphorylated glyceric acids. J. Biol. Chem. 234, 469–471.

Correll, C.C., Munishkin, A., Chan, Y.L., Ren, Z., Wool, I.G., Steitz, T.A., 1998. Crystal structure of the ribosomal RNA domain essential for binding elongation factors. Proc. Natl. Acad. Sci. U. S. A. 95, 13436–13441.

Correll, C.C., Wool, I.G., Munishkin, A., 1999. The two faces of the *Escherichia coli* 23 S rRNA Sarcin/Ricin domain: the structure at 1.11 A resolution. J. Mol. Biol. 292, 275–287.

Chan, Y.L., Endo, Y., Wool, I.G., 1983. The sequence of the nucleotides at the α-sarcin cleavage site in rat 28 S ribosomal ribonucleic acid. J. Biol. Chem. 258, 12768–12770.

De Antonio, C., Martínez-del-Pozo, A., Mancheño, J.M., Oñaderra, M., Lacadena, J., Martínez-Ruiz, A., Pérez-Cañadillas, J.M., Bruix, M., Gavilanes, J.G., 2000. Assignment of the contribution of the tryptophan residues to the spectroscopic and functional properties of the ribotoxin α-sarcin. Proteins 41, 350–361.

DeLano, W.L., 2008. ThePyMOL Molecular Graphics System. San Diego, California.

Dey, P., Tripathi, M., Batra, J.K., 2007. Involvement of loops l2 and L4 of ribonucleolytic toxin restrictocin in its functional activity. Protein Pept. Lett. 14, 125–129.

Endo, Y., Chan, Y.-L., Lin, A., Tsurugi, K., Wool, I., 1988. The cytotoxins α-sarcin and ricin retain their specificity when tested on a synthetic oligoribonucleotide (35-mer) that mimics a region of 28 S ribosomal ribonucleic acid. J. Biol. Chem. 263, 7917–7920.

Endo, Y., Huber, P.W., Wool, I.G., 1983. The ribonuclease activity of the cytotoxin α-sarcin. The characteristics of the enzymatic activity of α-sarcin with ribosomes and ribonucleic acids as substrates. J. Biol. Chem. 258, 2662–2667.

García-Mayoral, F., García-Ortega, L., Álvarez-García, E., Bruix, M., Gavilanes, J.G., Martínez-del-Pozo, A., 2005. Modeling the highly specific ribotoxin recognition of ribosomes. FEBS Lett. 579, 6859–6864.

García-Ortega, L., Alvarez-Garcia, E., Gavilanes, J.G., Martinez-del-Pozo, A., Joseph, S., 2010. Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. Nucleic Acids Res. 38, 4108–4119.

García-Ortega, L., Lacadena, J., Lacadena, V., Masip, M., de Antonio, C., Martínez-Ruiz, A., Martínez-del-Pozo, A., 2000. The solubility of the ribotoxin α-sarcin, produced as a recombinant protein in *Escherichia coli*, is increased in the presence of thioredoxin. Lett. Appl. Microbiol. 30, 298–302.

García-Ortega, L., Lacadena, J., Mancheño, J.M., Oñaderra, M., Kao, R., Davies, J., Olmo, N., Martínez-del-Pozo, A., Gavilanes, J.G., 2001. Involvement of the amino-terminal β-hairpin of the *Aspergillus* ribotoxins on the interaction with membranes and nonspecific ribonuclease activity. Protein Sci. 1 (0), 1658–1668.

García-Ortega, L., Lacadena, J., Villalba, M., Rodríguez, R., Crespo, J.F., Rodríguez, J., Pascual, C., Olmo, N., Oñaderra, M., Martínez-del-Pozo, A., Gavilanes, J.G., 2005. Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Asp f 1 displaying reduced IgE binding. FEBS J. 272, 2536–2544.

García-Ortega, L., Masip, M., Mancheño, J.M., Oñaderra, M., Lizarbe, M.A., García-Mayoral, M.F., Bruix, M., Martínez-del-Pozo, A., Gavilanes, J.G., 2002. Deletion of the NH_2-terminal β-hairpin of the ribotoxin α-sarcin produces a nontoxic but active ribonuclease. J. Biol. Chem. 277, 18632–18639.

Gasset, M., Mancheño, J.M., Lacadena, J., Turnay, J., Olmo, N., Lizarbe, M.A., Martínez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1994. α-Sarcin, a ribosome-inactivating protein that translocates across the membrane of phospholipid vesicles. Curr. Top. Pept. Protein Res. 1, 99–104.

Gasset, M., Mancheño, J.M., Laynez, J., Lacadena, J., Fernández-Ballester, G., Martínez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1995. Thermal unfolding of the cytotoxin α-sarcin: phospholipid binding induces destabilization of the protein structure. Biochim. Biophys. Acta 1252, 126–134.

Gasset, M., Martínez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1989. Study of the interaction between the antitumour protein α-sarcin and phospholipid vesicles. Biochem. J. 258, 569–575.

Gasset, M., Oñaderra, M., Goormaghtigh, E., Gavilanes, J.G., 1991a. Acid phospholipid vesicles produce conformational changes on the antitumour protein α-sarcin. Biochim. Biophys. Acta 1080, 51–58.

Gasset, M., Oñaderra, M., Martínez-del-Pozo, A., Schiavo, G.P., Laynez, J., Usobiaga, P., Gavilanes, J.G., 1991b. Effect of the antitumour protein α-sarcin on the thermotropic behavour of acid phospholipid vesicles. Biochim. Biophys. Acta 1068, 9–16.

Gasset, M., Oñaderra, M., Thomas, P.G., Gavilanes, J.G., 1990. Fusion of phospholipid vesicles produced by the anti-tumour protein α-sarcin. Biochem. J. 265, 815–822.

Glück, A., Wool, I.G., 2002. Analysis by systematic deletion of amino acids of the action of the ribotoxin restrictocin. Biochim. Biophys. Acta 1594, 115 126.

Hebert, E.J., Giletto, A., Sevcik, J., Urbanikova, L., Wilson, K.S., Dauter, Z., Pace, C.N., 1998. Contribution of a conserved asparagine to the conformational stability of ribonucleases Sa, Ba, and T1. Biochemistry 38, 16192–16200.

Herrero-Galán, E., García-Ortega, L., Olombrada, M., Lacadena, J., Martínez-del-Pozo, A., Gavilanes, J.G., Oñaderra, M., 2013. Hirsutellin A: a paradigmatic example of the insecticidal function of fungal ribotoxins. Insects 4, 339–356.

Herrero-Galán, E., Lacadena, J., Martínez-del-Pozo, A., Boucias, D.G., Olmo, N., Oñaderra, M., Gavilanes, J.G., 2008. The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. Proteins 72, 217–228.

Kao, R., Davies, J., 1999. Molecular dissection of mitogillin reveals that the fungal ribotoxins are a family of natural genetically engineered ribonucleases. J. Biol. Chem. 274, 12576–12582.

Kao, R., Davies, J., 2000. Single amino acid substitutions affecting the specificity of the fungal ribotoxin mitogillin. FEBS Lett. 466, 87–90.

Kao, R., Martinez-Ruiz, A., Martinez del Pozo, A., Crameri, R., Davies, J., 2001. Mitogillin and related fungal ribotoxins. Methods Enzymol. 341, 324–335.

Korennykh, A.V., Correll, C.C., Piccirilli, J.A., 2007. Evidence for the Importance of Electrostatics in the Function of Two Distinct Families of Ribosome Inactivating Toxins. RNA 13: 1391–1396.

Korennykh, A.V., Piccirilli, J.A., Correll, C.C., 2006. The electrostatic character of the ribosomal surface enables extraordinarily rapid target location by ribotoxins. Nat. Struct. Mol. Biol. 13, 436–443.

Lacadena, J., Álvarez-García, E., Carreras-Sangrà, N., Herrero-Galán, E., Alegre-Cebollada, J., García-Ortega, L., Oñaderra, M., Gavilanes, J.G., Martínez-del-Pozo, A., 2007. Fungalribotoxins: molecular dissection of a family of natural killers. FEMS Microbiol. Rev. 31, 212–237.

Lacadena, J., Mancheño, J.M., Martínez-Ruiz, A., Martínez-del-Pozo, A., Gasset, M.,

Oñaderra, M., Gavilanes, J.G., 1995. Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin. Biochem. J. 309, 581–586.
Lacadena, J., Martínez-del-Pozo, A., Barbero, J.L., Mancheño, J.M., Gasset, M., Oñaderra, M., López-Otín, C., Ortega, S., García, J.L., Gavilanes, J.G., 1994. Overproduction and purification of biologically active native fungal α-sarcin in. E. Coli. Gene. 142, 147–151.
Lacadena, J., Martinez del Pozo, A., Martinez-Ruiz, A., Perez-Canadillas, J.M., Bruix, M., Mancheño, J.M., Onaderra, M., Gavilanes, J.G., 1999. Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin alpha-sarcin. Proteins 37, 474–484.
Mancheño, J.M., Gasset, M., Albar, J.P., Lacadena, J., Martinez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1995. Membrane interaction of a β-structure-forming synthetic peptide comprising the 116–139th sequence region of the cytotoxic protein α-sarcin. Biophys. J. 68, 2387–2395.
Mancheño, J.M., Gasset, M., Lacadena, J., Ramón, F., Martínez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1994. Kinetic study of the aggregation and lipid mixing produced by α-sarcin on phosphatidylglycerol and phosphatidylserine vesicles: stopped-flow light scattering and fluorescence energy transfer measurements. Biophys. J. 67, 1117–1125.
Mancheño, J.M., Martínez-del-Pozo, A., Albar, J.P., Oñaderra, M., Gavilanes, J.G., 1998. A peptide of nine amino acid residues from α-sarcin cytotoxin is a membrane-perturbing structure. J. Pept. Res. 51, 142–148.
Martínez-del-Pozo, A., Gasset, M., Oñaderra, M., Gavilanes, J.G., 1988. Conformational study of the antitumor protein α-sarcin. Biochim. Biophys. Acta 953, 280–288.
Martínez-Ruiz, A., García-Ortega, L., Kao, R., Lacadena, J., Oñaderra, M., Mancheño, J.M., Davies, J., Martínez-del-Pozo, A., Gavilanes, J.G., 2001. RNase U2 and α-sarcin: a study of relationships. Methods Enzymol. 341, 335–351.
Mazet, I., Vey, A., 1995. Hirsutellin A, a toxic protein produced *in vitro* by *Hirsutella thompsonii*. Microbiology 141, 1343–1348.
Olombrada, M., Herrero-Galán, E., Tello, D., Oñaderra, M., Gavilanes, J.G., Martinez-del-Pozo, A., García-Ortega, L., 2013. Fungal extracellular ribotoxins as insecticidal agents. Insect Biochem. Mol. Biol. 43, 39–46.
Olombrada, M., Martínez-del-Pozo, A., Medina, P., Budia, F., Gavilanes, J.G., García-Ortega, L., 2014a. Fungal ribotoxins: natural protein-based weapons against insects. Toxicon 83, 69–74.
Olombrada, M., Rodríguez-Mateos, M., Prieto, M., Pla, J., Remacha, M., Martínez-del-Pozo, A., Gavilanes, J.G., Ballesta, J.P.G., García-Ortega, L., 2014b. The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by α-sarcin of the eukaryotic ribosome. Biochemistry 53, 1545–1547.
Oñaderra, M., Mancheño, J.M., Gasset, M., Lacadena, J., Schiavo, G., Martínez-del-Pozo, A., Gavilanes, J.G., 1993. Translocation of α-sarcin across the lipid bilayer of asolectin vesicles. Biochem. J. 295, 221–225.
Pérez-Cañadillas, J.M., Campos-Olivas, R., Lacadena, J., Martínez-del-Pozo, A., Gavilanes, J.G., Santoro, J., Rico, M., Bruix, M., 1998. Characterization of pKa values and titration shifts in the cytotoxic ribonuclease α-sarcin by NMR. Relationship between electrostatic interactions, structure, and catalytic function. Biochemistry 37, 15865–15876.
Pérez-Cañadillas, J.M., Guenneugues, M., Campos-Olivas, R., Santoro, J., Martínez del Pozo, A., Gavilanes, J.G., Rico, M., Bruix, M., 2002. Backbone dynamics of the cytotoxic Ribonuclease α-sarcin by ^{15}N NMR relaxation methods. J. Biomol. NMR 24, 301–316.
Pérez-Cañadillas, J.M., Santoro, J., Campos-Olivas, R., Lacadena, J., Martínez-del-Pozo, A., Gavilanes, J.G., Rico, M., Bruix, M., 2000. The highly refined solution structure of the cytotoxic ribonuclease α-sarcin reveals the structural requirements for substrate recognition and ribonucleolytic activity. J. Mol. Biol. 299, 1061–1073.
Plantinga, M.J., Korennykh, A.V., Piccirilli, J.A., Correll, C.C., 2008. Electrostatic interactions guide the active site face of a structure-specific ribonuclease to its RNA substrate. Biochemistry 47, 8912–8918.
Plantinga, M.J., Korennykh, A.V., Piccirilli, J.A., Correll, C.C., 2011. The ribotoxin restrictocin recognizes its RNA substrate by selective engagement of active site residues. Biochemistry 50, 3004–3013.
Siemer, A., Masip, M., Carreras, N., García-Ortega, L., Oñaderra, M., Bruix, M., Martínez-del-Pozo, A., Gavilanes, J.G., 2004. Conserved asparagine residue 54 of α-sarcin plays a role in protein stability and enzyme activity. Biol. Chem. 385, 1165–1170.
Tello, D., Rodríguez-Rodriguez, M., Yélamos, B., Gómez-Gutiérrez, J., Ortega, S., Pacheco, B., Peterson, D.L., Gavilanes, F., 2010. Expression and structural properties of a chimeric protein based on the ectodomains of E1 and E2 hepatitis C virus envelope glycoproteins. Protein Expr. Purif. 71, 123–131.
Viegas, A., Herrero-Galán, E., Oñaderra, M., Macedo, A.L., Bruix, M., 2009. Solution structure of hirsutellin A. New insights into the active site and interacting interfaces of ribotoxins. FEBS J. 276, 2381–2390.
Wool, I., 1996. Extraribosomal functions of ribosomal proteins. Trends Biochem. Sci. 21, 164–165.
Wool, I.G., 1997. Structure and mechanism of action of the cytotoxic ribonuclease α-sarcin. In: D'Alessio, G., Riordan, J.F. (Eds.), Ribonucleases. Academic Press, San Diego, California, pp. 131–162.
Yang, X., Gerczei, T., Glover, L., Correll, C.C., 2001. Crystal structures of restrictocin-inhibitor complexes with implications for RNA recognition and base flipping. Nat. Struct. Biol. 8, 968–973.
Yang, X.J., Moffat, K., 1996. Insights into specificity of cleavage and mechanism of cell entry from the crystal structure of the highly specific *Aspergillus* ribotoxin, restrictocin. Structure 4, 837–852.

Supplementary Material

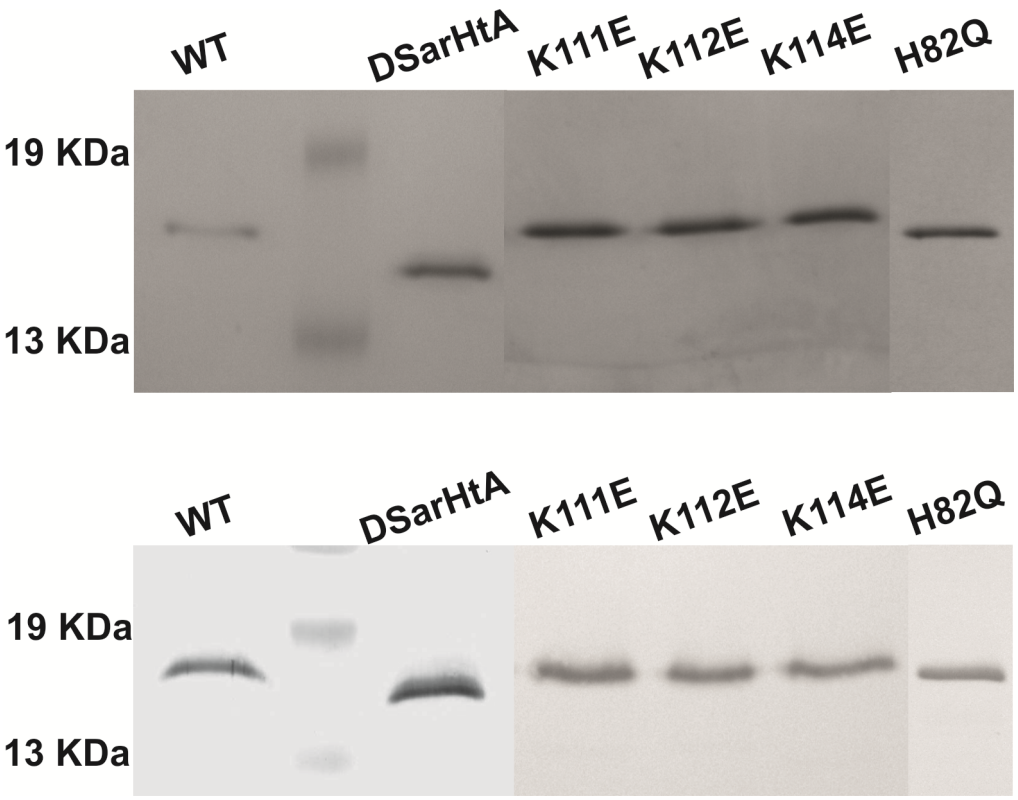
Table S1. Coding nucleotide 5'→3' sequences of the primers used to construct the different mutants studied in this work, with the nucleotide changes or insertion underlined. In addition to the oligoribonucleotides shown, in all cases it was also used another one containing the corresponding complementary sequence.

α-Sarcin mutant	**Nucleotide sequence**
K111E	5'-AGTTTGATTCGGAGAAGCCCAAGGA-3'
K112E	5'-TTGATTCGAAGGAGCCCAAGGAAAA-3'
K114E	5'-CGAAGAAGCCCGAGGAAAATCCTGG-3'
H82Q	5'-GACCGTCCTCCCAAGCAGAGCAAGGACGGAAAC-3'
ΔSarHtA	5'-TCCCAGTGTGACCGTGGCGATGCTATCCTGCTGGAGTTCCCA-3'

Table S2. Purification yield, expressed as milligrams of protein isolated from one liter of original culture, and spectroscopic parameters of α-sarcin wild-type and the different mutants studied. Fluorescence emission relative quantum yields (Q_{Trp} and Q_{Tyr}) are referred to the values of the wild-type protein. T_m values are also shown.

Protein	**Purification yield**	$E^{0.1\%}_{(280nm,\ 1cm)}$	Q_{Trp}	Q_{Tyr}	T_m (ºC)
α-sarcin	7.0	1.34	1.00	1.00	52.0
K111E	7.5	1.39	0.85	0.73	51.0
K112E	10.8	1.37	1.19	1.10	53.0
K114E	6.0	1.35	1.19	1.05	49.0
H82Q	1.0	1.51	6.65	1.54	45.0
ΔSarHtA	6.0	1.58	5.46	1.79	48.0

Figure S1. SDS–PAGE analysis (*upper panel*) and Western blot identification (*lower panel*) of the wild-type α-sarcin and the different mutants studied. BioRad prestained 13 and 19 KDa molecular weight standards (second line from left) are also indicated. All samples were boiled in the presence of 5.0% β-mercaptoethanol before being loaded onto the gel.



A2. Participación de la horquilla β amino-terminal y las lisinas del bucle 5 de la ribotoxina hirsutelina A en su actividad insecticida

Después de la α-sarcina, una de las ribotoxinas más estudiadas es la hirsutelina A (HtA), producida por el hongo entomopatógeno *Hirsutella thompsonii*. Las ribotoxinas poseen una elevada identidad tanto de secuencia como de estructura no sólo cuando se comparan entre ellas, sino también cuando esta comparación se lleva a cabo con otras RNasas fúngicas no tóxicas, como la RNasa T1. La mayor parte de las diferencias observadas entre las ribotoxinas y las RNasas no tóxicas se concentra en la longitud y carga de sus bucles, así como en la horquilla β amino-terminal. HtA posee un tamaño intermedio entre la mayoría de las ribotoxinas y las RNasas no tóxicas, pero aún así alberga en su pequeña estructura todas las características de las ribotoxinas. Su extremo amino-terminal presenta diferencias notables con el de la α-sarcina en términos de longitud y carga. En este trabajo se han producido, purificado y caracterizado cuatro mutantes de la HtA que afectan a la horquilla β amino-terminal (la deleción Δ[8-15]) y al bucle 5, muy próximo en la estructura a la región anterior (K115E, K118E y K123E). Los resultados apuntan a que el centro activo de HtA es más adaptable que el de la α-sarcina, siendo capaz de acomodar cambios tanto electrostáticos como estructurales que no serían posibles en el caso de las ribotoxinas de mayor tamaño. De este modo se explica que las variantes que se estudian aquí sólo presenten pequeñas diferencias en cuanto a la actividad ribonucleolítica y a la citotoxicidad frente a células de insecto en cultivo.

Trabajo A2: Olombrada M, García Ortega L, Lacadena J, Oñaderra M, Gavilanes JG y Martínez del Pozo, A (2015). "Involvement of loop 5 lysine residues and the N-terminal β-hairpin of the ribotoxin Hirsutellin A on its insecticidal activity."Enviado.

Involvement of loop 5 lysine residues and the N-terminal β-hairpin of the ribotoxin hirsutellin A on its insecticidal activity.

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Running Head: Insecticidal role of HtAβ-hairpin and loop 5

Abstract

Ribotoxins are cytotoxic members of the family of fungal extracellular ribonucleases best represented by RNase T1. They share a high degree of sequence identity and a common structural fold, including the geometric arrangement of their active sites. However, ribotoxins are larger, with a well-defined N-terminal β-hairpin, and display longer and positively charged unstructured loops. These structural differences account for their cytotoxic properties. Unexpectedly, the discovery of hirsutellin A (HtA), a ribotoxin produced by the invertebrate pathogen *Hirsutella thompsonii*, showed how it was possible to accommodate these features into a shorter amino acid sequence. Examination of HtA N-terminal β-hairpin reveals differences in terms of length, charge, and spatial distribution. Consequently, four different HtA mutants were prepared and characterized. One of them was the result of deleting this hairpin [Δ(8-15)] while the other three affected single Lys residues in its close spatial proximity (K115E, K118E, and K123E). The results obtained support the general conclusion that HtA active site would show a high degree of plasticity, being able to accommodate electrostatic and structural changes not suitable for the other previously known larger ribotoxins, as the variants described here only presented small differences in terms of ribonucleolytic activity and cytotoxicity against cultured insect cells.

Keywords: Ribotoxins, hirsutellin A, Ribonucleases, rRNA

Abbreviations: HtA, hirsutellin A; Δ(8-15), a mutant version of HtA where residues 8 to 15 (PKLDGREK) have been substituted by two Gly; PPE, poison primer extension; RIP, ribosome-inactivating protein; RNase, ribonuclease; SRL, sarcin–ricin loop.

Introduction

Ribotoxins are cytotoxic members of the family of fungal extracellular ribonucleases (RNases) best represented by RNase T1 (Yoshida 2001). They have been

shown to be extremely toxic because they exert their ribonucleolytic activity just on a unique phosphodiester bond of the larger molecule of rRNA in the ribosome, leading to protein synthesis inhibition and cell death (Lacadena et al. 2007).This rRNA bond is unique because it is located at an evolutionarily conserved site, the sarcin-ricin loop (SRL), with essential roles in ribosome function (García-Ortega et al. 2010) and maturation (Lo et al. 2010), and it is also the target of the family of plant ribosome-inactivating proteins (RIPs), a group of glycosidases best represented by ricin (Nielsen and Boston 2001).

In addition to their ribonucleolytic activity, fungal ribotoxins have the ability to cross lipid membranes in the absence of any known protein receptor, mainly due to their ability to interact with acid phospholipids(Gasset et al. 1994; Martínez-Ruiz et al. 2001; Oñaderra et al. 1993). This feature is the explanation of why these proteins display a remarkable but not highly specific antitumoral activity (Jennings et al. 1965; Lacadena et al. 2007; Olmo et al. 2001; Olson and Goerner 1965; Turnay et al. 1993).

α-Sarcin is the most extensively characterized ribotoxin (Lacadena et al. 2007), but many others have been identified and/or characterized in different fungal species (Huang et al. 1997; Lin et al. 1995; Martínez-Ruiz et al. 1999a; Martínez-Ruiz et al. 1999b; Parente et al. 1996; Varga and Samson 2008; Wirth et al. 1997). Most of them show a high degree of conservation with sequence identities above 60% and even higher than 85% in many instances. The only exception known so far is hirsutellin A (HtA), another extracellular RNase produced by an invertebrate pathogen, the fungus *Hirsutella thompsonii*, which has been demonstrated to be a ribotoxin even though it only displays about 25% sequence identity with the previously known members of the family (Figure 1) (Boucias et al. 1998; Herrero-Galán et al. 2013; Herrero-Galán et al. 2008; Martínez-Ruiz et al. 1999b; Olombrada et al. 2014a). HtA is considerably smaller than the rest of ribotoxins known but still contains the same elements of periodic secondary structure and an identical structural arrangement of the active site residues (Figure 1) (Herrero-Galán et al. 2012a; Herrero-Galán et al. 2012b; Herrero-Galán et al. 2008; Martínez-Ruiz et al. 1999b; Viegas et al. 2009). HtA is indeed a well-known insecticidal protein (Boucias et al. 1998; Liu et al. 1995). Therefore, the characterization of HtA as a fungal ribotoxin not only proved that the unique abilities of ribotoxins can be accommodated into a shorter amino acid sequence (Herrero-Galán et al. 2008), but also suggested that they might play an insecticidal role in nature (Olombrada et al. 2013; Olombrada et al. 2014a). Consequently, deciphering the distinct molecular features that enable rather different proteins like α-sarcin and HtA to show this identical and singular toxic behavior is of great interest and might be useful in the design and development of new and effective biotechnological tools for different applications like new biopesticides (Olombrada et al. 2013; Olombrada et al. 2014a), the study of ribosome-related diseases (De la Cruz et al. 2015; Olombrada et al. 2014b) or the construction of new immunotoxins (Tomé-Amat et al. 2015).

The N-terminal β-hairpin of ribotoxins has been shown to modulate their catalytic activity (García-Ortega et al. 2001; García-Ortega et al. 2002). Deletion variants of α–sarcin and Aspf1 (another well-known ribotoxin), in which this hairpin had been eliminated without affecting the overall three-dimensional structure of the proteins (García-Mayoral et al. 2004; García-Ortega et al. 2005b; García-Ortega et al. 2002), retained their nonspecific ribonucleolytic activity as well as their ability to specifically cleave SRL-like oligonucleotides. However, these deletion mutants were not able to inactivate rabbit ribosomes with the high specificity and efficiency displayed by the wild-type proteins and, therefore, were much less cytotoxic (García-Ortega et al. 2005b; García-Ortega et al. 2002; Olombrada et al. 2014b). Comparison of α-sarcin and HtA three-dimensional structures revealed major differences precisely in the sequence, length, and conformation of their N-terminal β-hairpins (Fig. 1A), suggesting that an additional protein region would be required for HtA specific recognition of the ribosome. Within this idea, close inspection of these structures suggested that this function could be assisted by the presence of a cluster of Lys residues along HtA loop 5 (Figure 1B) (Herrero-Galán et al. 2012a). Therefore, and in order to investigate this matter, the work presented here deals with the production and characterization of a deletion mutant version of HtA, where amino acids 8 to 15 (PKLDGREK) were substituted by two Gly residues [Δ(8-15)], and three single mutants affecting the mentioned Lys residues, which were changed to Glu (K115E, K118E, and K123E).

Results

Protein purification and spectroscopic characterization

All four HtA mutants studied were purified to homogeneity according to their SDS-PAGE behavior. Amino acid analyses were in good agreement with the mutation produced. These analysis and the corresponding UV-absorption spectra were used to calculate their $E^{0.1\%}$ (280 nm, 1 cm) values (Table 1). These values were very similar for all of them in accordance with the fact that there were neither Trp nor Tyr residues among the mutated amino acids. Furthermore, this invariance of the $E^{0.1\%}$ values suggested a conservation of their three-dimensional structures. In agreement with this observation, the far-UV CD spectra of the four mutant proteins (Fig. 2), as well as their fluorescence emission spectra (Fig. 3), showed only very minor changes.

Higher differences were observed in terms of thermostability (Table 1). The Δ(8-15) protein showed a decrease of 12ºC in its T_m value, and the Lys to Glu mutants displayed reductions of only 3-5 degrees respect to the wild-type HtA (Table 1). Nevertheless, the functional differences observed for these proteins should not be attributed to the lower thermostability of the mutants, since the T_m values were far above the temperature values used in the experiments herein described.

HtA variant	Purification yield[a]	$E^{0.1\%}$ (280 nm, 0.1cm)	Tm (ºC)[b]
WT	1.00	2.00	62.0
Δ(8-15)	0.50	2.00	50.0
K115E	0.36	2.01	57.0
K118E	0.60	1.87	59.0
K123E	0.45	1.90	58.5

Table 1. Some features of the purified proteins

[a]Milligrams of protein obtained per liter of original culture.

[b]Mid-point temperature of the thermal denaturation transition measured as the ellipticity value variation at 215 nm as a function of temperature (García-Mayoral et al. 2006; Herrero-Galán et al. 2008; Lacadena et al. 1995; Lacadena et al. 1999).

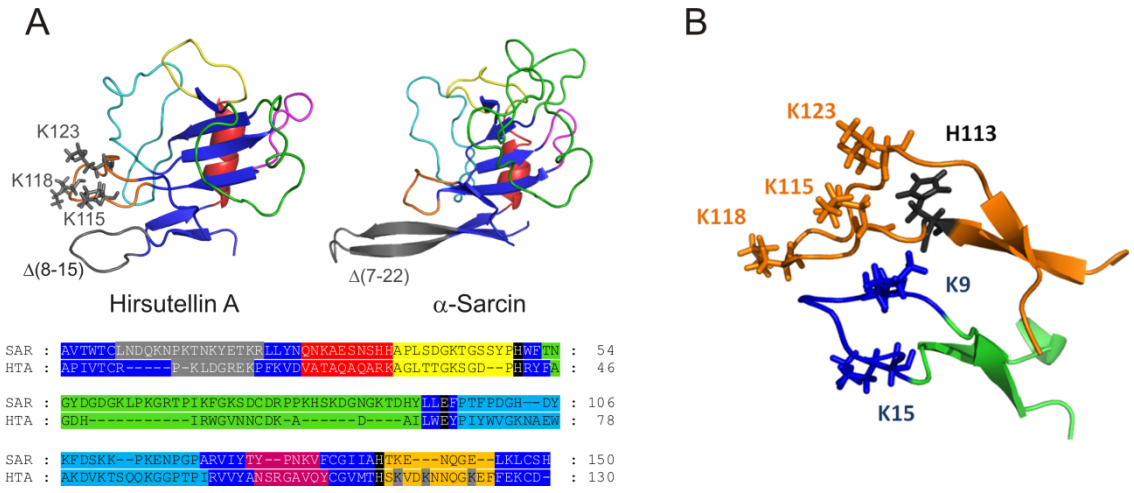


Figure 1. (A) Diagrams showing the three-dimensional structures of HtA (PDB ID: 2KAA) and α-sarcin (PDB ID: 1DE3) as well as the alignment of their amino acid sequences. Side chains of the residues mutated in HtA are shown in gray, according to the sequence alignment shown below. The N-terminal β-hairpin backbone stretch of both proteins, substituted by two Gly residues in the Δ(8-15) mutant, is also shown in gray. The rest of the backbone color codes are blue for the NH_2-terminal β-hairpin and the β-strands; red for the α-helix; yellow for loop 1; green for loop 2; light blue for loop 3,magenta for loop 4; orange for loop 5. These color codes are also maintained along the sequence alignment. (B) Diagram showing the spatial distribution of loop 5 (orange) and the N-terminal β–hairpin (blue) of HtA. Side chains of HtA Lys 9, 15, 115, 118, and 123, and catalytic His 113 (Herrero-Galán et al. 2012a; Viegas et al. 2009) are shown. Diagrams were generated with PyMol software (DeLano 2008).

Ribonucleolytic activity against eukaryotic ribosomes

The natural optimum substrates of ribotoxins are eukaryotic ribosomes. Accordingly, the enzymatic characterization of both wild-type and mutant proteins was first performed following the release of the characteristic rRNA α-fragment resulting from their cleaving activity on the larger ribosomal subunit of ribosomes of a cell-free rabbit reticulocyte lysate. As it can be observed in Fig. 4A, although all mutant proteins assayed retained this specific ribonucleolytic activity against rabbit ribosomes, it was

slightly impaired when compared to the wild-type protein (Fig. 4B). This effect was especially evident for the K123E and ∆(8-15) variants which showed much lower activity values at the lower enzyme concentrations employed.

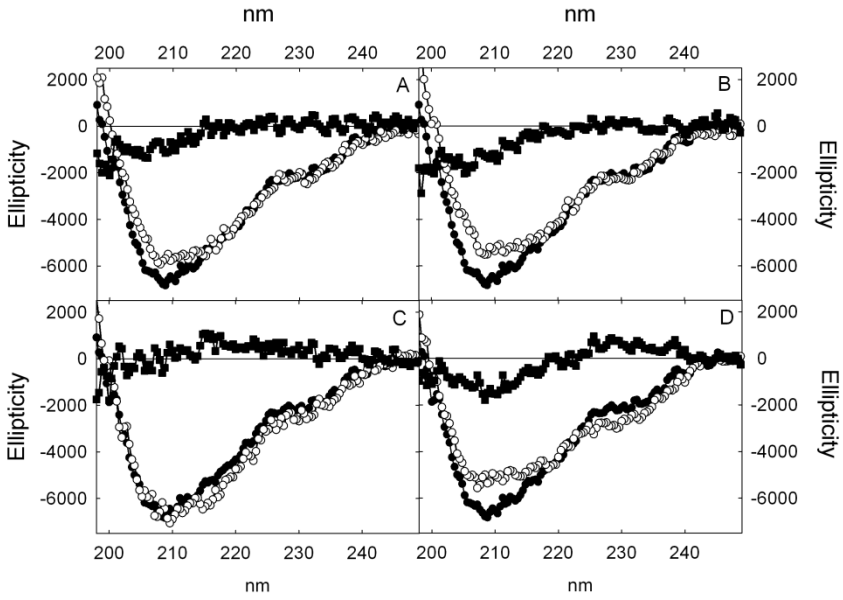
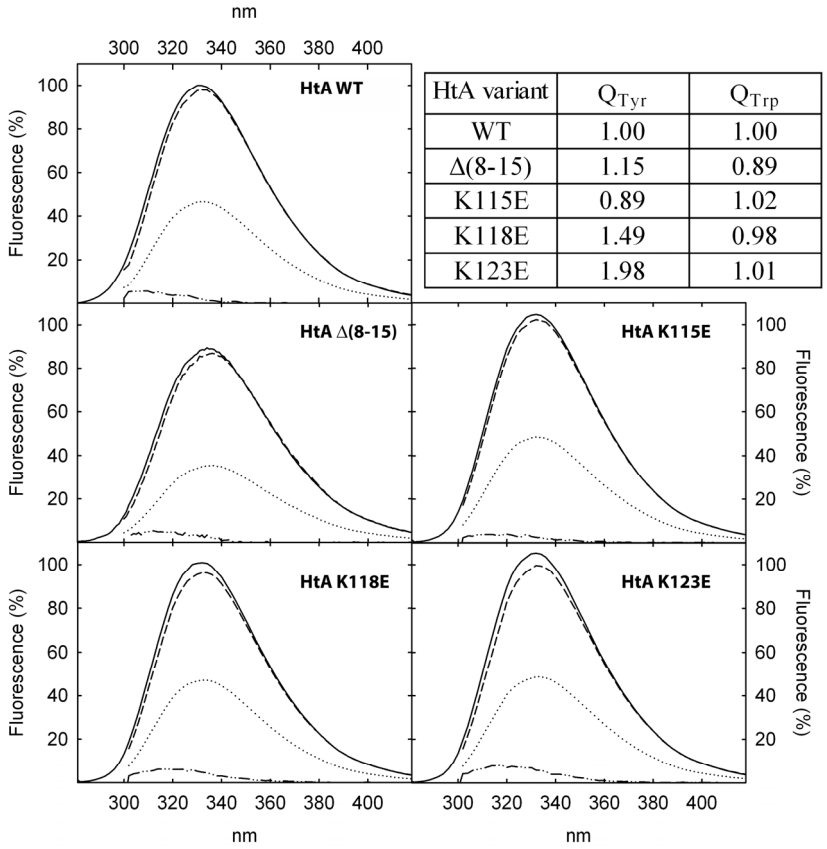


Figure 2.Far-UV circular dichroism spectra of wild type HtA (black dots) and its different mutants (white dots): K115E (A), K118E, (B), K123E (C), and Δ(8-15) (D).The line with black squares represents the calculated difference spectra wild-type minus mutant. Circular dichroism values are expressed as mean residue weight ellipticities (θ_{MRW}) in units of degree x cm^2 x $dmol^{-1}$.

Ribonucleolytic activity against an SRL-like oligonucleotide

Short oligoribonucleotides mimicking the SRL sequence and structure (SRL-like oligos) are frequently used to evaluate the influence of other ribosomal elements on ribotoxins activity. Thus, although ribotoxins cleave these SRL-like oligos specifically, producing only two smaller fragments which can be fractionated on a polyacrylamide gel, this activity is several orders of magnitude less efficient than that one produced on intact ribosomes (Endo et al. 1988; Gluck and Wool 1996; Kao et al. 2001; Wool 1997; Wool et al. 1992). It is assumed that this is due to the absence of additional interactions with specific ribosomal regions such as some large subunit conserved proteins (García-Mayoral et al. 2005) as well as the contribution of electrostatic interactions with the complete ribosome (Korennykh et al. 2007). In this occasion, and in good accordance with the assays using intact ribosomes described above, all mutants studied alsoretained the ability to specifically cleave the SRL-like oligo employed (Fig. 5A). In fact, the K115E variant displayed a cleaving pattern which was practically indistinguishable from that shown by wild-type HtA. K123E and ∆(8-15) were also the least efficient variants, showing again much lower activity values than wild-type HtA(Fig. 5B) at the lower protein concentrations assayed.



HtA variant	Q_{Tyr}	Q_{Trp}
WT	1.00	1.00
Δ(8-15)	1.15	0.89
K115E	0.89	1.02
K118E	1.49	0.98
K123E	1.98	1.01

Figure 3. Fluorescence emission spectra of wild-type HtA and the mutant variants studied. All spectra were recorded at identical protein concentrations. Spectra were recorded using 275 (continuous lines) and 295 (dotted lines) nm as excitation wavelengths. These spectra were normalized at wavelengths above 380 nm to obtain the tryptophan contribution (dashed lines). Finally, tyrosine contribution was calculated as described in the Materials and Methods section (dashed-dotted lines). Fluorescence emission units were arbitrary, and referred to the maximum value of wild-type HtA upon excitation at 275 nm. The table shows the relative fluorescence Tyr or Trp quantum yield for excitation at 275 nm to that of wild-type HtA considered as 1.00

Toxic effect on insect cell lines in culture

It is well known the dramatic effect of HtA on the inhibition of *in vivo S. frugiperda* cells protein biosynthesis (Castaño-Rodríguez et al. 2015; Herrero-Galán et al. 2013; Olombrada et al. 2013). Therefore, this insect cellular line was chosen to evaluate the toxic effect of the mutants studied when employed against intact cells. In good accordance with the previous results shown above, K115E and K118E showed very similar insecticidal behavior as wild-type HtA, at least in terms of their ability to inhibit protein biosynthesis in *S. frugiperda* cultured cells (Fig. 6). On the other hand, K123E and Δ(8-15) showed around a ten-fold increase in the value of protein concentration needed to produce a 50% protein biosynthesis inhibition (Fig. 6). That is to say, these two mutants displayed lower cytotoxic activity against insect cells in comparison with the behavior shown by wild-type HtA.

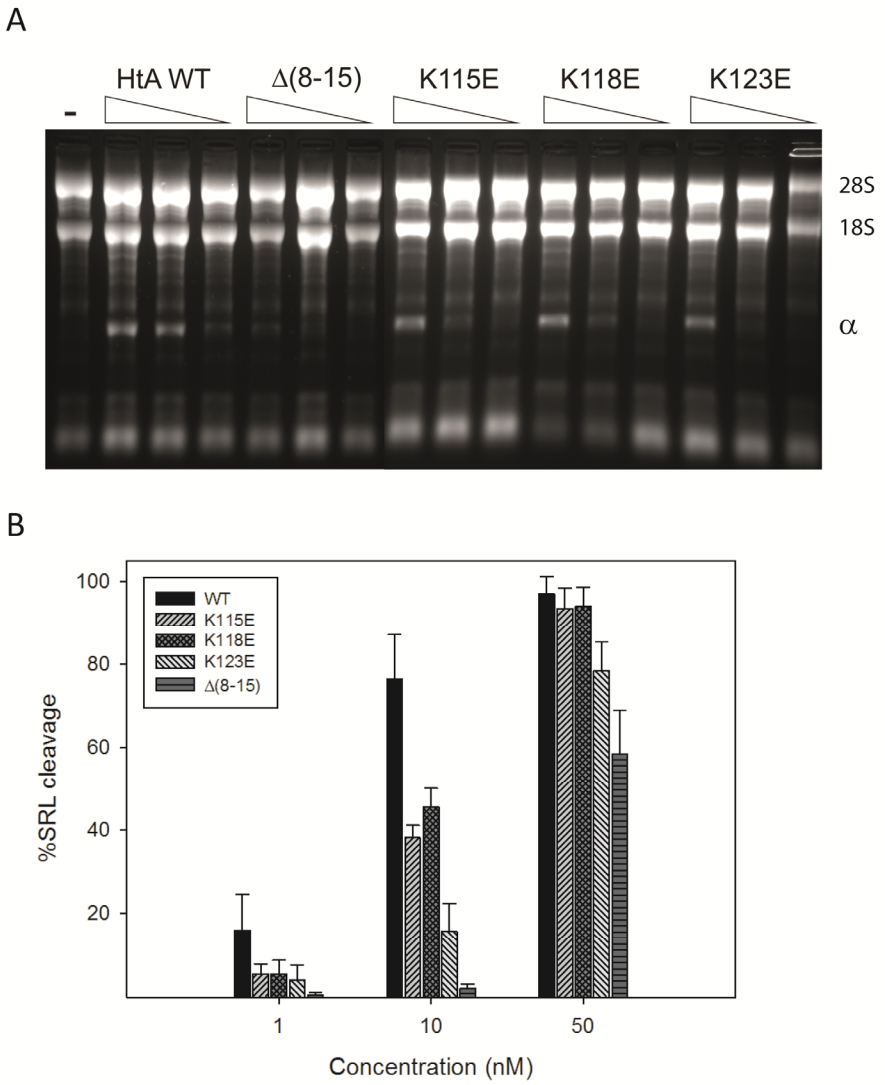


Figure 4. (A) Ribonucleolytic activity assay of wild-type HtA and the four mutants studied as shown by the release of the 400-nucleotide α-fragment (which position is indicated) from the 28 S rRNA of rabbit ribosomes. (B) Quantitation of this assay by poison primer extension. Results are expressed as the percentage of SRL cleavage versus the concentration of ribotoxin assayed. Wild-type and mutant proteins were assayed at 1.0, 10.0 and 50.0 nM concentrations. Means are calculated from the result of at least three independent experiments.

Discussion

RNase T1 is not only one of the most exhaustively characterized proteins but also the best known member of the family of fungal extracellular RNases (Loverix and Steyaert 2001; Yoshida 2001), a group that includes ribotoxins (Lacadena et al. 2007; Martínez-Ruiz et al. 2001). All members of this family, ribotoxins included, share a high degree of sequence identity (Arruda et al. 1990; Fernández-Luna et al. 1985; López-Otín et al. 1984; Martínez-Ruiz et al. 1999a; Martínez-Ruiz et al. 1999b; Rodríguez et al. 1982; Sacco et al. 1983; Wirth et al. 1997) and a common structural fold concerning the architecture and connectivity of the regular secondary structure elements (Campos-Olivas et al. 1996; Martínez-Ruiz et al. 2001; Pérez-Cañadillas et al. 2000;

Viegas et al. 2009; Yang and Moffat 1996) and the geometric arrangement of the residues involved in the active site (Martínez-Ruiz et al. 2001; Pérez-Cañadillas et al. 2000; Viegas et al. 2009). However, only ribotoxins show cytotoxic properties. Non-toxic fungal extracellular RNases are smaller than ribotoxins, lack a well-defined N-terminal β-hairpin, and display much shorter and negatively charged unstructured loops (Lacadena et al. 2007; Pace et al. 1991). Accordingly, it was predicted that these structural differences would account for the cytotoxic properties of ribotoxins (Martínez-del-Pozo et al. 1988).

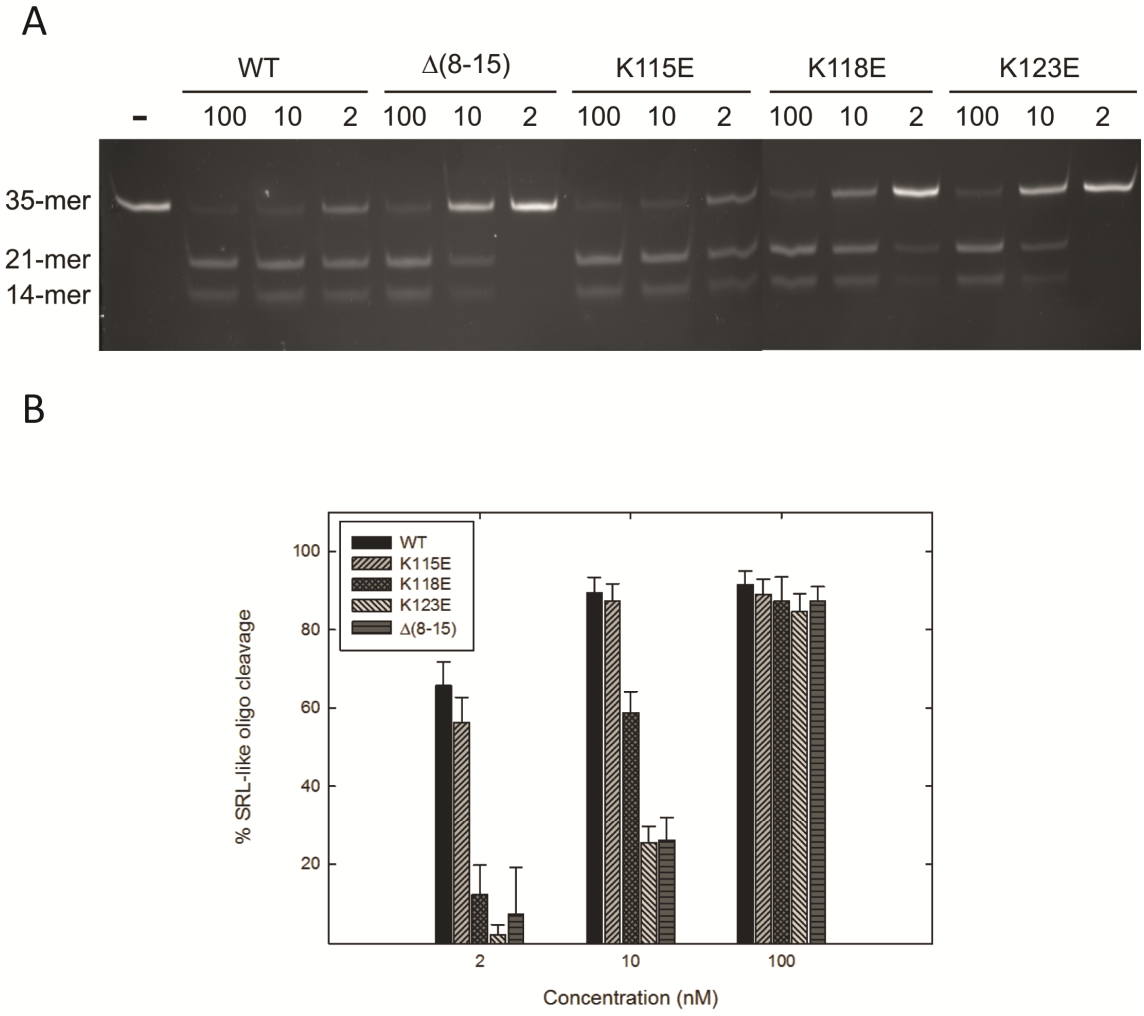


Figure 5.(A) Activity assay on a 35-mer oligonucleotide mimicking the SRL. This substrate was incubated in presence of wild-type, Δ(8-15), K115E, K118E, and K123E versions of HtA. A control in the absence of enzyme is also shown (-). The positions of the 21-mer and 14-mer fragments resulting from the specific cleavage of a single phosphodiester bond and of the intact 35-mer oligo are indicated. Proteins were assayed at 2.0, 10.0 and 100.0 nM concentrations. (B) Quantitation of these activities shown as percentage of SRL cleavage as a function of the protein concentration assayed.

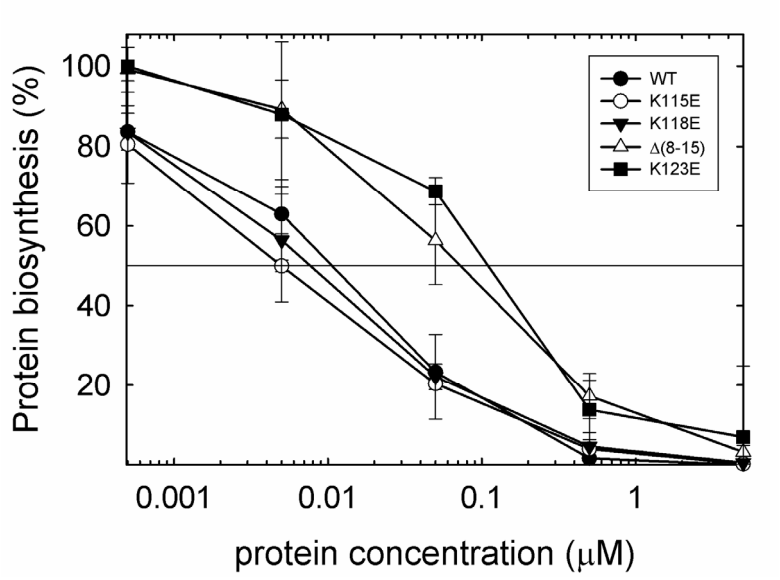


Figure 6. Protein biosynthesis ± SD in Sf9 insect cells cultured in the presence of different ribotoxin variants concentrations. Results shown are the average of three independent sets of experiments. A thin horizontal line has been drawn to indicate 50% of protein synthesis inhibition.

These predictions, which were formulated at the early stages of the study of ribotoxins (Lamy et al. 1992; Mancheño et al. 1995; Martínez-del-Pozo et al. 1988), have been confirmed since then (Campos-Olivas et al. 1996; García-Mayoral et al. 2005; García-Mayoral et al. 2004; García-Ortega et al. 2001; García-Ortega et al. 2005b; García-Ortega et al. 2002).For example, the N-terminal β-hairpins of α-sarcin, mitogillin, restrictocin, and Aspf1 (four well characterized ribotoxins)modulate their catalytic activity(García-Ortega et al. 2001; García-Ortega et al. 2005b; García-Ortega et al. 2002; Kao and Davies 1999; Kao and Davies 2000). These studies included deletion variants in which the hairpin had been eliminated without affecting the overall three-dimensional structure of the proteins (García-Mayoral et al. 2004; García-Ortega et al. 2005b; García-Ortega et al. 2002). Deletion mutants [α-sarcin Δ(7-22) and Aspf1 Δ(7-22)] which retained their non-specific ribonucleolytic activity as well as their ability to cleave SRL-like oligonucleotides, but were not able to specifically inactivate rabbit ribosomes. Therefore, they were much less cytotoxic (García-Ortega et al. 2005b; García-Ortega et al. 2002).Docking studies revealed that this N-terminal β-hairpin could establish interactions with ribosomal proteins in order to direct the ribotoxin to the SRL region of the ribosome (García-Mayoral et al. 2005).

HtA was discovered and characterized as a new and singular fungal ribotoxin (Boucias et al. 1998; Herrero-Galán et al. 2008; Liu et al. 1995). It is around 20 residues shorter than all other known fungal ribotoxins (Martínez-Ruiz et al. 1999a; Martínez-Ruiz et al. 1999b) and displays sequence identities of only about 25% (Martínez-Ruiz et al. 1999b). Again, most of the amino acid differences are located at the protein unstructured loops and the N-terminal β-hairpin (Fig. 1B). The length of the N-terminal β-hairpin in HtA is also intermediate between those in RNase T1 and α-sarcin, having

20 amino acids in HtA, 26 in α-sarcin and 12in RNase T1.A closer examination of α-sarcin and HtA hairpin sequences reveals another remarkable difference in terms of available charges, since two of the positively charged residues in α-sarcin are missing in HtA (Fig. 1A). From a functional point of view, this reduction in length and charge would be extremely important given the role of this protein region in ribotoxins cytotoxic activity (Álvarez-García et al. 2009b; García-Ortega et al. 2001; García-Ortega et al. 2005b; García-Ortega et al. 2002; Olombrada et al. 2014b).

The aim of the present work was to investigate the role of HtA N-terminal β-hairpin on itsribonucleolytic activity and its cytotoxic behavior. Within this idea, the Δ(8-15) mutant was produced and purified. Moreover, comparison of α-sarcin and HtA loop 5 (Fig. 1A), in close proximity to the N-terminal β-hairpin, revealed how it is longer in HtA and contains precisely two additional Lys residues (K118 and K123).In fact, loop 5 in HtA adopts a different orientation, pointing towards the closed end of the shorter hairpin. This could allow loop 5, which includes three lysine residues, K115 (conserved in α-sarcin), K118 and K123, to compensate for the lack of charge on that face of the molecule (Viegas et al. 2009). These residues could eventually fulfill the role of the two Lys missing along the hairpin when compared to α-sarcin's sequence. Accordingly, the K115E, K118E, and K123E mutants were also produced, purified, and characterized.

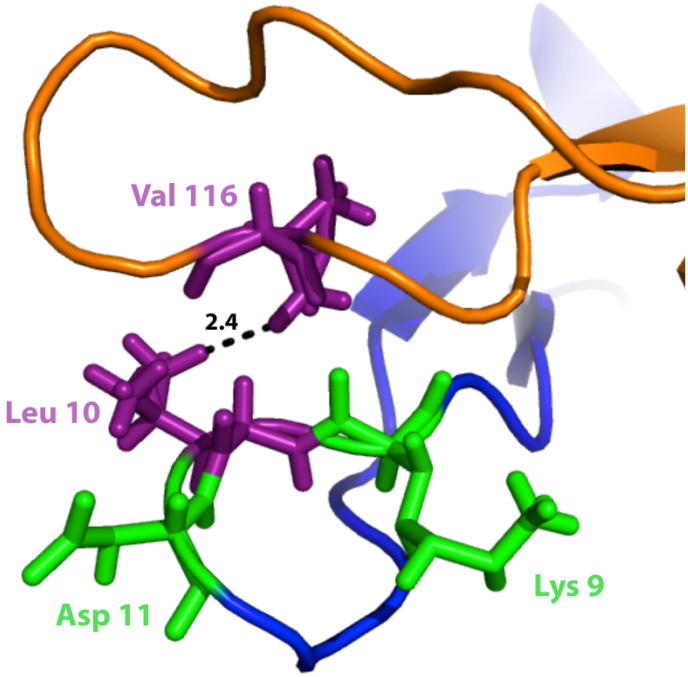


Figure 7. Three-dimensional structure representation of the hydrophobic interaction established between HtA Leu 10 and Val 116 residues.

All four mutants retained the overall features of the wild-type protein as revealed by their far-UV CD (Fig. 2) and fluorescence emission (Fig. 3) spectra. In fact, from a structural point of view the only major difference observed was the high reduction of 12ºC in the T_m value of Δ(8-15) (Table 1). A reduction in clear contrast

with the results obtained for the other equivalent deletion mutants studied before, α-sarcin Δ(7-22) and Aspf1 Δ(7-22), where reductions of only 2.0 and 4.4 degrees were obtained, respectively (García-Ortega et al. 2005b; García-Ortega et al. 2002).Close inspection of HtA three-dimensional structure reveals a hydrophobic interaction between Leu 10 and Val 116 (Fig. 7) which would be eliminated in the deletion mutant. The absence of this interaction could explain the observed T_m value reduction (Table 1).

In the two deletion mutants previously studied, α-sarcin Δ(7-22) and Aspf1 Δ(7-22), the two proteins did not recognize the SRL within the context of intact ribosomes under the assay conditions employed.However, they retained their ribonucleolytic activity against less specific substrates such as SRL-like oligonucleotides (García-Ortega et al. 2005b; García-Ortega et al. 2002). The results presented now reveal that the four HtA mutants studied showed a decreased ability to generate the α-fragment when assayed against rabbit ribosomes, but all they still retained the specificity (Fig. 4). In this case, however, the results obtained with the 35 mer SRL-like oligonucleotide assays were very similar (Fig. 5). Overall, these results suggest that for HtA not only the N-terminal β-hairpin but also loop 5 residues are involved in ribosome recognition and that, as predicted, loop 5 would contribute to specificity since a missing N-terminal β-hairpin does not render a non-specific RNase.

It has been also proposed that the enzymatic efficiency of α-sarcin is dependent on the interactions between its catalytic His-137, loop 5 residues, and the N-terminal β-hairpin (Álvarez-García et al. 2009a; Masip et al. 2001; Pérez-Cañadillas et al. 2000). After more than two decades of systematic studies, it has also become well-established that the activity of ribotoxins is extremely dependent on electrostatic interactions among their active site residues (Álvarez-García et al. 2006; Masip et al. 2001; Pérez-Cañadillas et al. 1998; Pérez-Cañadillas et al. 2000). His 42, Glu 66 and His 113 form the catalytic triad of HtA (Fig. 1), in good accordance with many other fungal ribonucleases, toxic or not (Lacadena et al. 2007; Lacadena et al. 1995; Lacadena et al. 1998; Lacadena et al. 1999; Yoshida 2001). However, single mutants of this catalytic triad of HtA (Herrero-Galán et al. 2012a) maintained to some extent the ability to degrade eukaryotic ribosomes, so none of these three residues is strictly essential for the ribonucleolytic activity of this protein on this substrate (Herrero-Galán et al. 2012a).On the other hand, none of the single mutants of the catalytic triad of HtA was able to cleave the synthetic SRL (Herrero-Galán et al. 2012a). These results showed the importance of a microstructural environment more than the presence of a particular individual residue in the activity of HtA. The results presented now with this new set of mutants are in perfect agreement with this hypothesis, and suggest that this microenvironment is influenced by interactions beyond the active site. Overall, HtA seems to be a more adaptable enzyme, with the ability to accommodate changes which would not be suitable for the larger ribotoxins characterized before. In this

regard, it could be considered a better fitted enzyme for the purpose of inactivating ribosomes in more variable environments.

In addition to their ribonucleolytic activity, ribotoxins cross lipid membranes, showing a cytotoxic behavior against different types of cells. Of special relevance is their toxic effect against insect cells, an evidence of their potential insecticidal behavior in nature (Olombrada et al. 2013; Olombrada et al. 2014a). The results reported now would be in agreement with the observation that HtA Lys 115 and 118 residues do not play a major role in recognizing the ribosome (Figs. 4 and 5) supporting their unaltered cytotoxic activity (Fig 6). On the other hand,K123E and Δ(8-15) showed a significantly impaired cytotoxic activity (Fig. 6) which also correlated with the detrimental ribonucleolytic activities that they displayed against ribosomes and the SRL-like oligonucleotide (Figs. 4 and 5). For these two mutants, we cannot discard however a role for the residues mutated in lipid interaction and passage across cell membranes as an additional explanation of their lower cytotoxicity. In fact, it has been reported before how α-sarcin Lys 14 and 21, with identical amino acids in equivalent positions of HtA (Fig. 1), are crucial residues for the correct achievement of these interactions (Álvarez-García et al. 2009b).

In summary, the results obtained support the general conclusion that, in spite of its smaller size, HtA active site would be highly adaptable, accommodating changes which would not be suitable for the larger ribotoxins characterized before. This flexibility makes HtA active site susceptible to intramolecular interactions with the N-terminal β-hairpin and loop 5.In addition, the hydrophobic interaction between Leu 10 and Val 116 seems to be crucial for the high protein thermostability. Finally, not only the N-terminal β-hairpin but also Lys 123 in loop 5 play an important role for this protein to exert its insecticidal action on cultured insect cells.

Experimental procedures

DNA manipulation

All materials and reagents were of molecular biology grade. Cloning procedures, PCR-based oligonucleotide site-directed mutagenesis, and bacterial manipulations were carried out as previously described (Álvarez-García et al. 2006; Castaño-Rodríguez et al. 2015; Herrero-Galán et al. 2012a; Herrero-Galán et al. 2012b; Lacadena et al. 1994; Martínez-Ruiz et al. 2001). Mutagenesis constructions were performed using different sets of complementary mutagenic primers (Table S1). Mutations were confirmed by DNA sequencing at the corresponding Complutense University facility. The plasmid used as template for mutagenesis, containing the cDNA sequence of wild-type HtA, had already been described (Herrero-Galán et al. 2012a; Herrero-Galán et al. 2012b; Herrero-Galán et al. 2013; Herrero-Galán et al. 2008).

Protein production and purification

Production and purification of HtA mutants was carried out as previously described for the wild-type recombinant protein (Herrero-Galán et al. 2008). Briefly, plasmid pTacTacHtA was used for protein production in *Escherichia coli* BL21 (DE3) cells, induced with 1 mM IPTG at 37°C for 4 h. Purification using a low percentage Ni^{2+}nitrilotriacetic acid agarose affinity column allowed elution of the different mutants with 10 mM MOPS buffer (pH 7.8), containing 200 mM imidazole. SDS-PAGE analysis, protein hydrolysis, and amino acid analysis were performed according to standard procedures, also as previously described (Lacadena et al. 1994; Martínez-Ruiz et al. 2001).

Spectroscopic characterization

Spectroscopic characterization was performed following well-established procedures (Álvarez-García et al. 2006; Álvarez-García et al. 2009b; García-Ortega et al. 2005a; García-Ortega et al. 2001; García-Ortega et al. 2002; Lacadena et al. 1999; Martínez-Ruiz et al. 2001). Absorbance measurements were carried out on a Beckman DU640 spectrophotometer (Beckman Coulter, Brea, CA, USA) at 200 nm/min scanning speed and room temperature. Amino acid analyses and the corresponding UV-absorbance spectra were also used to calculate their extinction coefficients (Table 1). Circular dichroism spectra were obtained in a Jasco 715 spectropolarimeter (Jasco, Easton, MD, USA), equipped with a thermostated cell holder and a Neslab-111 circulating water bath, at 0.2 nm/s scanning speed. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 215 nm in the 25–80 °C range using a rate of temperature increment of 30ºC per hour. Fluorescence emission spectra were recorded on an SLM Aminco8000 spectrofluorimeter at 25°C using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission beams, respectively, were used. All these experiments were performed in 50 mM sodium phosphate, pH 7.0, containing 0.1 M NaCl.

Ribonucleolytic activity assays

All procedures were carried out using RNase-free materials and reagents. The specific ribonucleolytic activity of ribotoxins is usually detected by the release of a 400-nt fragment (*α-fragment*) from eukaryotic ribosomes (Schindler and Davies 1977). Therefore, HtA and its mutants were assayed against ribosomes contained in a rabbit cell-free reticulocyte lysate (Kao et al. 2001). After a 15 min incubation at room temperature of the sample with 1-50 nM of the different proteins in 23 mM Tris-HCl,

23 mM KCl, 6 mM EDTA pH 7.5, RNA was phenol-extracted, precipitated with isopropanol and visualized by ethidium bromide staining after electrophoresis on denaturing 2.4 % agarose gels as described (Herrero-Galán et al. 2008; Lacadena et al. 1994; Lacadena et al. 1999).

Ribotoxin cleavage was quantitatedby poison primer extension (PPE) as described (García-Ortega et al. 2010; Olombrada et al. 2014b). Reverse transcription where dATP is substituted by ddATP was performed using the complementary sequence downstream the SRL in the 28S rRNA of *O. cuniculus* (5'-ACCAAATGTCTGAACCTGCGG-3'). The products of this reverse transcription were then separated in a denaturing 10% polyacrylamide gel and the amount of ^{32}P present in each one of the DNA bands produced was quantitated using a PhosphorImager screen (Molecular Dynamics).

Finally, cleavage of a synthetic oligonucleotide that mimics the sequence and structure of the SRL was also measured for HtA and its mutant variants. Synthesis of this SRL-like 35mer RNA was performed as previously described (García-Ortega et al. 2010; Kao et al. 2001). SRL (2 μM) was incubated with 2-100 nM protein for 15 min at 37^{o}C in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MgCl_2$. Reaction products were run on a denaturing 19 % (w/v) polyacrylamide gel and visualized by ethidium bromide staining.

Insect cell culture and toxicity assays

The insect cell line *Spodoptera frugiperda* (Sf9) was cultured at 27^{o}C as described (Castaño-Rodríguez et al. 2015; Olombrada et al. 2013) in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker) as indicated by the manufacturer. Protein solutions were prepared in culture medium and sterilized by ultrafiltration. Protein biosynthesis assays were carried out by seeding Sf9 cells into 24-well plates at a cell density of 10^5 cells per well, maintaining themunder standard culture conditions up to 80% confluency. Then, monolayer cultures were incubated in 0.5 mL of fresh medium with serial dilutions of ribotoxin from 5.0 μM to 0.5 nM final concentrations. Following 18 h of incubation at 27^{o}C medium was replaced by culture medium supplemented with 0.5 μCi/well of [^{3}H]-leucine. After 5 h of incubation medium was removed and cell protein content was precipitated with 5 % trichloroacetic acid and washed three times with ethanol. The precipitate was dried, dissolved in 200 μL of 0.1 N NaOH, 0.1% SDS and radioactivity was measured in a Beckman LS 3801 liquid scintillation counter. Results are expressed as percentage of incorporated radioactivity relative to samples without ribotoxin added.

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Supplementary Material

HtA mutant	Nucleotide sequence
K115E	5'- ATGACGCACAGCGAGGTCGACAAGAAT -3'
K118E	5'- ACAGCAAGGTCGACGAGAATAACCAGG-3'
K123E	5'- AATAACCAGGGCGAGGAGTTCTTTGAG -3'
Δ(8-15)	5'-GTCACCTGCCGGGGTGGTCCTTTCAAGGTAGACGTG -3'

Table S1. Coding nucleotide 5'→3' sequences of the primers used to construct the different mutants studied in this work, with nucleotide changes or insertions underlined. These and the corresponding complementary ones were used to obtain each mutation.

References

Álvarez-García, E, García-Ortega, L, De los Ríos, V, Gavilanes, JG and Martínez-del-Pozo, A (2009a) Influence of key residues on the heterologous extracellular production of fungal ribonuclease U2 in the yeast *Pichia pastoris*. *Protein Expr Purif* **65**: 223-9.

Álvarez-García, E, García-Ortega, L, Verdun, Y, Bruix, M, Martínez-del-Pozo, A and Gavilanes, JG (2006) Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of α-sarcin. *BiolChem* **387**: 535-41.

Álvarez-García, E, Martínez-del-Pozo, A and Gavilanes, JG (2009b) Role of the basic character of α-sarcin's NH_2-terminal β-hairpin in ribosome recognition and phospholipid interaction.*Arch Biochem Biophys* **481**: 37-44.

Arruda, LK, Platts-Mills, TA, Fox, JW and Chapman, MD (1990) *Aspergillus fumigatus* allergen I, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. *J Exp Med* **172**: 1529-1532.

Boucias, DG, Farmerie, WG and Pendland, JC (1998) Cloning and sequencing of cDNA of the insecticidal toxin hirsutellin A. *J Invertebr Pathol* **72**: 258-61.

Campos-Olivas, R, Bruix, M, Santoro, J, Martínez-del-Pozo, A, Lacadena, J, Gavilanes, JG and Rico, M (1996) Structural basis for the catalytic mechanism and substrate specificity of the ribonuclease α-sarcin.*FEBS Lett* **399**: 163-165.

Castaño-Rodríguez, C, Olombrada, M, Partida-Hanon, A, Lacadena, J, Oñaderra, M, Gavilanes, JG, García-Ortega, L and Martínez-del-Pozo, A (2015) Involvement of loops 2 and 3 of ?-sarcin on its ribotoxic activity. *Toxicon* **96C**: 1-9.

De la Cruz, J, Karbstein, K and Woolford, JL, Jr. (2015) Functions of ribosomal proteins in assembly of eukaryotic ribosomes *in vivo*. *Annu Rev Biochem* **84**: 93-129.

DeLano, WL (2008) ThePyMOL Molecular Graphics System. *San Diego, California*.

Endo, Y, Chan, Y-L, Lin, A, Tsurugi, K and Wool, I (1988) The cytotoxins α-sarcin and ricin retain their specificity when tested on a synthetic oligoribonucleotide (35-mer) that mimics a region of 28 S ribosomal ribonucleic acid. *J Biol Chem* **263**: 7917-7920.

Fernández-Luna, JL, López-Otín, C, Soriano, F and Méndez, E (1985) Complete amino acid sequence of the *Aspergillus* cytotoxin mitogillin. *Biochemistry* **24**: 861-867.

García-Mayoral, F, García-Ortega, L, Álvarez-García, E, Bruix, M, Gavilanes, JG and Martínez-del-Pozo, A (2005) Modeling the highly specific ribotoxin recognition of ribosomes. *FEBS Lett* **579**: 6859-64.

García-Mayoral, MF, García-Ortega, L, Lillo, MP, Santoro, J, Martínez-del-Pozo, A, Gavilanes, JG, Rico, M and Bruix, M (2004) NMR structure of the noncytotoxic α-sarcin mutant Δ(7-22): the importance of the native conformation of peripheral loops for activity. *Protein Sci* **13**: 1000-1011.

García-Mayoral, MF, Martínez-del-Pozo, A, Campos-Olivas, R, Gavilanes, JG, Santoro, J, Rico, M, Laurents, DV and Bruix, M (2006) pH-Dependent Conformational Stability of the Ribotoxin α-Sarcin and Four Active Site Charge Substitution Variants. *Biochemistry* **45**: 13705-13718.

García-Ortega, L, Álvarez-García, E, Gavilanes, JG, Martínez-del-Pozo, A and Joseph, S (2010) Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. *Nucleic Acids Res* **38**: 4108-19.

García-Ortega, L, de los Ríos, V, Martínez-Ruiz, A, Oñaderra, M, Lacadena, J, Martínez-del-Pozo, A and Gavilanes, JG (2005a) Anomalous electrophoretic behavior of a very acidic protein: ribonuclease U2. *Electrophoresis* **26**: 3407-13.

García-Ortega, L, Lacadena, J, Mancheño, JM, Oñaderra, M, Kao, R, Davies, J, Olmo, N, Martínez-del-Pozo, A and Gavilanes, JG (2001) Involvement of the amino-terminal β-hairpin of the *Aspergillus* ribotoxins on the interaction with membranes and nonspecific ribonuclease activity. *Protein Sci* **10**: 1658-68.

García-Ortega, L, Lacadena, J, Villalba, M, Rodríguez, R, Crespo, JF, Rodríguez, J, Pascual, C, Olmo, N, Oñaderra, M, Martínez-del-Pozo, A and Gavilanes, JG (2005b) Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Asp f 1 displaying reduced IgE binding. *FEBS J* **272**: 2536-44.

García-Ortega, L, Masip, M, Mancheño, JM, Oñaderra, M, Lizarbe, MA, García-Mayoral, MF, Bruix, M, Martínez-del-Pozo, A and Gavilanes, JG (2002) Deletion of the NH_2-terminal β-hairpin of the ribotoxin α-sarcin produces a nontoxic but active ribonuclease. *J Biol Chem* **277**: 18632-9.

Gasset, M, Mancheño, JM, Lacadena, J, Turnay, J, Olmo, N, Lizarbe, MA, Martínez-del-Pozo, A, Oñaderra, M and Gavilanes, JG (1994) α-Sarcin, a ribosome-inactivating protein that translocates across the membrane of phospholipid vesicles. *Curr Top Pept Protein Res* **1**: 99-104.

Gluck, A and Wool, IG (1996) Dependence of depurination of oligoribonucleotides by ricin A-chain on divalent cations and chelating agents. *Biochemistry and Molecular Biology International* **39**: 285-291.

Herrero-Galán, E, García-Ortega, L, Lacadena, J, Martínez-Del-Pozo, A, Olmo, N, Gavilanes, JG and Oñaderra, M (2012a) Implication of an Asp residue in the ribonucleolytic activity of hirsutellin A reveals new electrostatic interactions at the active site of ribotoxins. *Biochimie* **94**: 427-33.

Herrero-Galán, E, García-Ortega, L, Lacadena, J, Martínez-Del-Pozo, A, Olmo, N, Gavilanes, JG and Oñaderra, M (2012b) A non-cytotoxic but ribonucleolytically specific ribotoxin variant: implication of tryptophan residues in the cytotoxicity of hirsutellin A. *Biol Chem* **393**: 449-56.

Herrero-Galán, E, García-Ortega, L, Olombrada, M, Lacadena, J, Martínez-del-Pozo, A, Gavilanes, JG and Oñaderra, M (2013) Hirsutellin A: A Paradigmatic Example of the Insecticidal Function of Fungal Ribotoxins. *Insects* **4**: 339-356.

Herrero-Galán, E, Lacadena, J, Martínez-del-Pozo, A, Boucias, DG, Olmo, N, Oñaderra, M and Gavilanes, JG (2008) The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. *Proteins* **72**: 217-28.

Huang, K-C, Hwang, Y-Y, Hwang, L and Lin, A (1997) Characterization of a new ribotoxin gene (c-sar) from *Aspergillus clavatus*. *Toxicon* **35**: 383-392.

Jennings, JC, Olson, BH, Roga, V, Junek, AJ and Schuurmans, DM (1965) α-Sarcin, a New Antitumor Agent. II. Fermentation and Antitumor Spectrum. *Appl Microbiol* **13**: 322-326.

Kao, R and Davies, J (1999) Molecular dissection of mitogillin reveals that the fungal ribotoxins are a family of natural genetically engineered ribonucleases. *J Biol Chem* **274**: 12576-12582.

Kao, R and Davies, J (2000) Single amino acid substitutions affecting the specificity of the fungal ribotoxin mitogillin. *FEBS Letters* **466**: 87-90.

Kao, R, Martínez-Ruiz, A, Martínez del Pozo, A, Crameri, R and Davies, J (2001) Mitogillin and related fungal ribotoxins. *Methods Enzymol* **341**: 324-35.

Korennykh, AV, Correll, CC and Piccirilli, JA (2007) Evidence for the importance of electrostatics in the function of two distinct families of ribosome inactivating toxins. *RNA* **13**: 1391-1396.

Lacadena, J, Álvarez-García, E, Carreras-Sangrà, N, Herrero-Galán, E, Alegre-Cebollada, J, García-Ortega, L, Oñaderra, M, Gavilanes, JG and Martínez-del-Pozo, A (2007) Fungal ribotoxins: molecular dissection of a family of natural killers. *FEMS Microbiol Rev* **31**: 212-237.

Lacadena, J, Mancheño, JM, Martínez-Ruiz, A, Martínez-del-Pozo, A, Gasset, M, Oñaderra, M and Gavilanes, JG (1995) Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin. *Biochem J* **309**: 581-586.

Lacadena, J, Martínez-del-Pozo, A, Barbero, JL, Mancheño, JM, Gasset, M, Oñaderra, M, López-Otín, C, Ortega, S, García, JL and Gavilanes, JG (1994) Overproduction and purification of biologically active native fungal α-sarcin in *Escherichia coli*. *Gene* **142**: 147-151.

Lacadena, J, Martínez-del-Pozo, A, Lacadena, V, Martínez-Ruiz, A, Mancheño, JM, Oñaderra, M and Gavilanes, JG (1998) The cytotoxin α-sarcin behaves as a cyclizing ribonuclease. *FEBS Lett* **424**: 46-48.

Lacadena, J, Martínez-del-Pozo, A, Martínez-Ruiz, A, Pérez-Cañadillas, JM, Bruix, M, Mancheño, JM, Oñaderra, M and Gavilanes, JG (1999) Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin α-sarcin.*Proteins* **37**: 474-84.

Lamy, B, Davies, J and Schindler, D (1992) The *Aspergillus* ribonucleolytic toxins (ribotoxins). In: *Genetically engineered toxins*. Frankel, AE, ed., pp. 237-258. Marcel Dekker, Inc., New York.

Lin, A, Huang, KC, Hwu, L and Tzean, SS (1995) Production of type II ribotoxins by *Aspergillus* species and related fungi in Taiwan. *Toxicon* **33**: 105-110.

Liu, W-Z, Boucias, DG and McCoy, CW (1995) Extraction and characterization of the insecticidal toxin hirsutellin A produced by *Hirsutella thompsonii* var. *thompsonii*. *Experimental Mycology* **19**: 254-262.

Lo, KY, Li, Z, Bussiere, C, Bresson, S, Marcotte, EM and Johnson, AW (2010) Defining the pathway of cytoplasmic maturation of the 60S ribosomal subunit. *Mol Cell* **39**: 196-208.

López-Otín, C, Barber, D, Fernández-Luna, JL, Soriano, F and Méndez, E (1984) The primary structure of the cytotoxin restrictocin. *Eur J Biochem* **143**: 621-634.

Loverix, S and Steyaert, J (2001) Deciphering the mechanism of RNase T1. *Methods Enzymol* **341**: 305-323.

Mancheño, JM, Gasset, M, Lacadena, J, Martínez-del-Pozo, A, Oñaderra, M and Gavilanes, JG (1995) Predictive study of the conformation of the cytotoxic protein α-sarcin: A structural model to explain α-sarcin-membrane interaction. *J Theor Biol* **172**: 259-67.

Martínez-del-Pozo, A, Gasset, M, Oñaderra, M and Gavilanes, JG (1988) Conformational study of the antitumor protein α–sarcin. *Biochim Biophys Acta* **953**: 280-8.

Martínez-Ruiz, A, García-Ortega, L, Kao, R, Lacadena, J, Oñaderra, M, Mancheño, JM, Davies, J, Martínez-del-Pozo, A and Gavilanes, JG (2001) RNase U2 and α-sarcin: A study of relationships. *Methods Enzymol* **341**: 335-51.

Martínez-Ruiz, A, Kao, R, Davies, J and Martínez-del-Pozo, A (1999a) Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. *Toxicon* **37**: 1549-63.

Martínez-Ruiz, A, Martínez-del-Pozo, A, Lacadena, J, Oñaderra, M and Gavilanes, JG (1999b) Hirsutellin A displays significant homology to microbial extracellular ribonucleases. *J Invertebr Pathol* **74**: 96-7.

Masip, M, Lacadena, J, Mancheño, JM, Oñaderra, M, Martínez-Ruiz, A, Martínez-del-Pozo, A and Gavilanes, JG (2001) Arginine 121 is a crucial residue for the specific cytotoxic activity of the ribotoxin α-sarcin. *Eur J Biochem* **268**: 6190-6.

Nielsen, K and Boston, RS (2001) Ribosome-inactivating proteins: A Plant Perspective. *Annu Rev Plant Physiol Plant Mol Biol* **52**: 785-816.

Olmo, N, Turnay, J, González de Buitrago, G, López de Silanes, I, Gavilanes, JG and Lizarbe, MA (2001) Cytotoxic mechanism of the ribotoxin α-sarcin. Induction of cell death via apoptosis. *Eur J Biochem* **268**: 2113-23.

Olombrada, M, Herrero-Galán, E, Tello, D, Oñaderra, M, Gavilanes, JG, Martínez-del-Pozo, A and García-Ortega, L (2013) Fungal extracellular ribotoxins as insecticidal agents. *Insect Biochem Mol Biol* **43**: 39-46.

Olombrada, M, Martínez-del-Pozo, A, Medina, P, Budia, F, Gavilanes, JG and García-Ortega, L (2014a) Fungal ribotoxins: Natural protein-based weapons against insects. *Toxicon* **83**: 69-74.

Olombrada, M, Rodríguez-Mateos, M, Prieto, D, Pla, J, Remacha, M, Martínez-del-Pozo, A, Gavilanes, JG, Ballesta, JP and García-Ortega, L (2014b) The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by α-sarcin of the eukaryotic ribosome. *Biochemistry* **53**: 1545-7.

Olson, BH and Goerner, GL (1965) α-Sarcin, a New Antitumor Agent. I. Isolation, Purification, Chemical Composition, and the Identity of a New Amino Acid. *Appl Microbiol* **13**: 314-321.

Oñaderra, M, Mancheño, JM, Gasset, M, Lacadena, J, Schiavo, G, Martínez-del-Pozo, A and Gavilanes, JG (1993) Translocation of α-sarcin across the lipid bilayer of asolectin vesicles. *Biochem J* **295**: 221-5.

Pace, CN, Heinemann, U, Hahn, U and Saenger, W (1991) Ribonuclease T1: Structure, function and stability. *Angew Chem Int Ed Engl* **30**: 343-360.

Parente, D, Raucci, G, Celano, B, Pacilli, A, Zanoni, L, Canevari, S, Adobati, E, Colnaghi, ML, Dosio, F, Arpicco, S, Cattel, L, Mele, A and De Santis, R (1996) Clavin a type-1 ribosome-inactivating protein from *Aspergillus clavatus* IFO 8605-cDNA isolation, heterologous expression, biochemical and biological characterization of the recombinant protein. *Eur J Biochem* **239**: 272-280.

Pérez-Cañadillas, JM, Campos-Olivas, R, Lacadena, J, Martínez-del-Pozo, A, Gavilanes, JG, Santoro, J, Rico, M and Bruix, M (1998) Characterization of pKa values and titration shifts in the cytotoxic ribonuclease α-sarcin by NMR. Relationship between electrostatic interactions, structure, and catalytic function. *Biochemistry* **37**: 15865-15876.

Pérez-Cañadillas, JM, Santoro, J, Campos-Olivas, R, Lacadena, J, Martínez-del-Pozo, A, Gavilanes, JG, Rico, M and Bruix, M (2000) The highly refined solution structure of the cytotoxic ribonuclease α-sarcin reveals the structural requirements for substrate recognition and ribonucleolytic activity. *J Mol Biol* **299**: 1061-73.

Rodríguez, R, López-Otín, C, Barber, D, Fernández-Luna, JL, González, G and Méndez, E (1982) Amino acid sequence homologies in α-sarcin, restrictocin and mitogillin. *Biochem Biophys Res Commun* **108**: 315-21.

Sacco, G, Drickamer, K and Wool, IG (1983) The primary structure of the cytotoxin α-sarcin. *J Biol Chem* **258**: 5811-5818.

Schindler, DG and Davies, JE (1977) Specific cleavage of ribosomal RNA caused by α-sarcin.*Nucleic Acids Res* **4**: 1097-1110.

Tomé-Amat, J, Herrero-Galán, E, Oñaderra, M, Martínez-Del-Pozo, A, Gavilanes, JG and Lacadena, J (2015) Preparation of an engineered safer immunotoxin against colon carcinoma based on the ribotoxin hirsutellin A. *FEBS J* **282**: 2131-2141.

Turnay, J, Olmo, N, Jiménez, A, Lizarbe, MA and Gavilanes, JG (1993) Kinetic Study of the Cytotoxic Effect of α-Sarcin, a Ribosome Inactivating Protein from *Aspergillus-giganteus*, on Tumour Cell Lines - Protein Biosynthesis Inhibition and Cell Binding. *Mol. Cell. Biochem.* **122**: 39-47.

Varga, J and Samson, RA (2008) Ribotoxin genes in isolates of *Aspergillus* section *Clavati*. *Antonie Van Leeuwenhoek* **94**: 481-5.

Viegas, A, Herrero-Galán, E, Oñaderra, M, Macedo, AL and Bruix, M (2009) Solution structure of hirsutellin A. New insights into the active site and interacting interfaces of ribotoxins. *FEBS J* **276**: 2381-90.

Wirth, J, Martínez-del-Pozo, A, Mancheño, JM, Martínez-Ruiz, A, Lacadena, J, Oñaderra, M and Gavilanes, JG (1997) Sequence determination and molecular characterization of gigantin, a cytotoxic protein produced by the mold *Aspergillus giganteus* IFO 5818. *Arch Biochem Biophys* **343**: 188-93.

Wool, IG (1997) Structure and mechanism of action of the cytotoxic ribonuclease α-sarcin. In: *Ribonucleases* (D'Alessio, G and Riordan, JF, eds.). pp. 131-162. Academic Press Inc, San Diego.

Wool, IG, Glück, A and Endo, Y (1992) Ribotoxin recognition of ribosomal RNA and a proposal for the mechanism of translocation. *Trends Biochem Sci* **17**: 266-269.

Yang, XJ and Moffat, K (1996) Insights into specificity of cleavage and mechanism of cell entry from the crystal structure of the highly specific *Aspergillus* ribotoxin, restrictocin. *Structure* **4**: 837-852.

Yoshida, H (2001) The ribonuclease T1 family. *Methods Enzymol* **341**: 28-41.

A3. Caracterización de una nueva toxina del hongo entomopatógeno *Metarhizium anisopliae*: la ribotoxina anisoplina

Metarhizium anisopliae es un hongo entomopatógeno de especial relevancia en biotecnología, ya que se utiliza en el control de plagas frente a multitud de ácaros e insectos como una alternativa a los pesticidas tradicionales. Además, se está investigando su uso como insecticida frente a uno de los mosquitos portadores de la malaria. Por ello, el estudio de los factores de virulencia de dicho hongo resulta de gran interés, sobre todo con el objetivo de mejorar sus propiedades biotecnológicas. La secuenciación del genoma de *M. anisopliae* ha desvelado la existencia de una proteína semejante a la hirsutelina A, una ribotoxina producida también por un hongo entomopatógeno, *Hirsutella thompsonii*. Las ribotoxinas fúngicas son una familia de proteínas tóxicas que poseen una tremenda especificidad y eficiencia frente a los ribosomas, de las que recientemente se han descrito además sus propiedades insecticidas. En este trabajo se describe la actividad característica de las ribotoxinas en cultivos del hongo *M. anisopliae*. Además, la anisoplina, como se ha llamado a esta nueva toxina, se ha clonado, expresado y purificado a homogeneidad como proteína recombinante en *Escherichia coli*. Esta nueva proteína comparte un 70% de identidad de secuencia con la ribotoxina HtA. La caracterización estructural y funcional de la anisoplina permite concluir que es una ribotoxina similar en estructura y termoestabilidad a la HtA, que posee la actividad ribonucleolítica específica frente a ribosomas típica de esta familia de proteínas, y propiedades citotóxicas frente a células de insecto, con una IC_{50} similar a la de las ribotoxinas. La descripción de esta nueva ribotoxina abre nuevas posibilidades biotecnológicas de diversa índole, aunque cómo y por qué usa *Metarhizium* esta toxina en la naturaleza es una cuestión todavía por resolver.

Trabajo A3: Olombrada M, Medina P, Budia F, Martínez del Pozo A y García Ortega L (2015) "Characterization of a new toxin from the entomopathogen fungus *Metarhizium anisopliae:* the ribotoxin anisoplin." En preparación.

Characterization of a new toxin from the entomopathogenic fungus *Metarhizium anisopliae*: the ribotoxin anisoplin.

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Keywords: hirsutellin, insecticide, *Metarhizium*, ribotoxin, sarcin.

Abreviations: CD, circular dichroism; HtA: hirsutellin A; PDB, potato dextrose broth; RIP, ribosome-inactivating protein; RNase, ribonuclease; SRL; sarcin/ricin loop.

Abstract

Metarhizium anisopliae is an entomopathogenic fungus of special relevance in biotechnology, with diverse applications in pest control. Several formulations are already commercialized and consist of a better alternative to classical pesticides. More interestingly, this fungus has been recently investigated as a control agent against adult malaria vectors. Therefore, studies of its virulence factors are of great interest in order to improve its biotechnological properties. Its sequenced genome pointed out a hypothetical hirsutellin-like protein, a ribotoxin. Ribotoxins are fungal extracellular and toxic ribonucleases with extraordinary specificity and efficiency against ribosomes in the target cell. Recently they have been described as potential insecticides but so far only one is produced by an entomopathogenic fungus. Here, we describe this ribotoxin characteristic activity in *M. anisopliae* cultures. Moreover, anisoplin, as we have named it, has been expressed and purified to homogeneity as a recombinant protein in *Escherichia coli*. Anisoplin is 70% identical to hirsutellin A (HtA), a ribotoxin from the entomopathogen *Hirsutella thompsonii*. Its structural and functional characterization, in comparison with HtA, has led to the conclusion that anisoplin is a secreted ribotoxin, similar in structure and thermostability to HtA (according to spectroscopical methods), with the unique ribonucleolytic activity against ribosomes typical of ribotoxins and cytotoxic properties against insect cells. How *Metarhizium* uses this toxin in nature and possible biotechnological applications are still interesting questions to work on.

Introduction

Entomopathogenic fungi are nowadays under scope in the search for alternative biopesticides needed for crop protection and other pests biocontrol. *Metarhizium anisopliae* and *Beauveria baussiana* are the most studied for these purposes, from their biological characterization under laboratory conditions to open field assays against different insects [1]. Even several formulations of these fungi are

already in the market designed to target a multitude of acari and insects like coleoptera and hemiptera [2]. Some interesting examples are the studies with *Metarhizium* to control the mite *Varroa destructor* in honey bees [3], and established commercial formulations like Bioblast® against termites (*Metarhizium*) or Mycotrol® against aphids and other organisms (*Beauveria*) [4].

Metarhizium was first employed by Elie Metchikoff in the late 1800s for biological control of wheat-grain beetles. Since then, biopesticides based on this fungus have greatly evolved. Even recently*, M. anisopliae* has become an interesting and promising alternative for the control of adult malaria vectors like the *Anopheles gambiae* mosquito since the appearance of resistant parasites and vectors to insecticides hampers the efforts to control the disease [4;5;6;7].

Due to its wide biotechnological applications, *M. anisopliae* has been extensively characterized in terms of its biology and pathogenesis, including the recent sequencing of its genome which constitutes an important resource to identify new virulence factors [8;9]. Regarding factors involved in its pathogenicity, the most studied ones are destruxins, ciclodepsipeptides also present in other insect pathogenic fungi [1;10]. Destruxins are responsible for several impairing functions over the target organism, mainly inhibition of the immune response. However, identification and characterization of new toxins will help, not only to better understand the biology of this fungus but also to provide new tools for the improvement of biopesticides. This should be an essential line of research because the Achilles' heel in the use of insect–pathogenic fungi for biocontrol is their speed of killing. Even when using a high dose of conidia it may take several days from the moment of contamination to penetrate the cuticle, multiply, intoxicate, and eventually kill the insect [11]. Detractors argue that the effect should be as immediate as possible, because in the pre-lethal (incubation) period these pests can still cause damage to crops or transmit disease. In the case of *Anopheles* mosquitoes, for example, this would be malaria parasite transmission. Therefore, one recent strategy with success in this regard consists in the genetic manipulation of fungal species to increase their virulence via expression of insecticidal protein/peptide toxins [12].

A well known family of toxic proteins secreted by fungi are ribotoxins, specific RNases against the large rRNA in the ribosome with lethal consequences for the target cell [13]. The main producer is *Aspergillus*, although the discovery of an entomopathogenic fungus, *Hirsutella thompsonii*, producing the ribotoxin hirsutellin A (HtA) suggests a wider distribution among fungi as well as their insecticide properties [14;15;16;17]. Ribotoxins are basic proteins of 130-150 amino acids able to cross lipid barriers and efficiently inactivate any kind of ribosome. In *Aspergillus,* they seem to be produced during conidia maturation, most probably as a defense mechanism against predators [18]. They have been deeply studied from a structural point of view as well as their molecular mechanism of protein synthesis inactivation [13;17;19;20]. More recently,

studies have begun to focus on their antitumor properties conjugated as immunotoxins and their toxicity against insects [16; 21; 22].

Results and discussion

Here we describe a new ribotoxin produced by *M. anisopliae* ARSEF23 as we found a "hirsutellin A toxin" gene (accession number MAA_10099) in its sequenced genome [8] during a Blast search for new ribotoxins launching HtA protein from *H. thompsonii* (accession number AAM95629) to the database (Fig. 1A). The sequence alignment of the potential ribotoxin (accession number EFY94422.1), named here as anisoplin, with the mature HtA showed 70% sequence identity between both proteins. Cisteines and active site residues described for HtA [14] were also conserved in anisoplin. Higher differences appeared in the signal peptides as it was expected. By comparison with HtA, and α-sarcin, the best characterized ribotoxin from *Aspergillus*, pre and pro peptides were assigned (Fig. 1A) [23;24;25;26;27]. Therefore, anisoplin seems to be an extracellular ribotoxin as well. Moreover, efficient compartmentalization and secretion by the producer fungus seems to be the best self-defense mechanism in ribotoxin production [24;27], so signal peptide in anisoplin might have evolved in this sense in *M. anisopliae*.

From an evolutionary point of view, the entomopathogens *Metarhizium* and *Hirsutella* are closer in the phylogenetic tree than *Aspergillus* [28]. It is not surprising then that anisoplin better resembles HtA than any other ribotoxin.

Another feature of the described anisoplin gene is an intron between 2nd and 3rd codons of the mature protein (accession number MAA_10099). Something similar occurs with the restrictocin gene, another well characterized ribotoxin [29]. This has been suggested as a strategy to attenuate protein toxicity [30], something that could be very useful for the producer fungus.

M. anisopliae ARSEF23 was studied for ribotoxin production *in vitro* growing it in liquid media as it was previously described for *A. giganteus* and *H. thompsonii,* producers of α-sarcin and HtA, respectively [14;31;32]. Culture progression was followed by pH measurements (Fig. 1B) and anisoplin production was analyzed by assaying the ribotoxin activity of the extracellular media (Fig. 1C). Fungal cultures in PDB and the ribotoxin production medium [32] were grown for 15 days (25ºC, 100 rpm) and the extracellular medium of different aliquots was assayed against reticulocytes to quantify the specific cleavage in the large rRNA characteristic of the ribotoxin action. Primer extension results showed the characteristic RNA fragment in both culture media from the first day of growth (Fig. 1C), independently of the culture progression measured by pH changes (Fig. 1B). This unique RNase activity is specific of ribotoxins, so this result constitutes the first evidence of a ribotoxin production by *M. anisopliae*. However, a protein band of the expected size was never found in SDS-PAGE concluding that anisoplin was produced but in minimal levels in the laboratory conditions employed. This behavior was different from that of *A. giganteus* which, in similar *in*

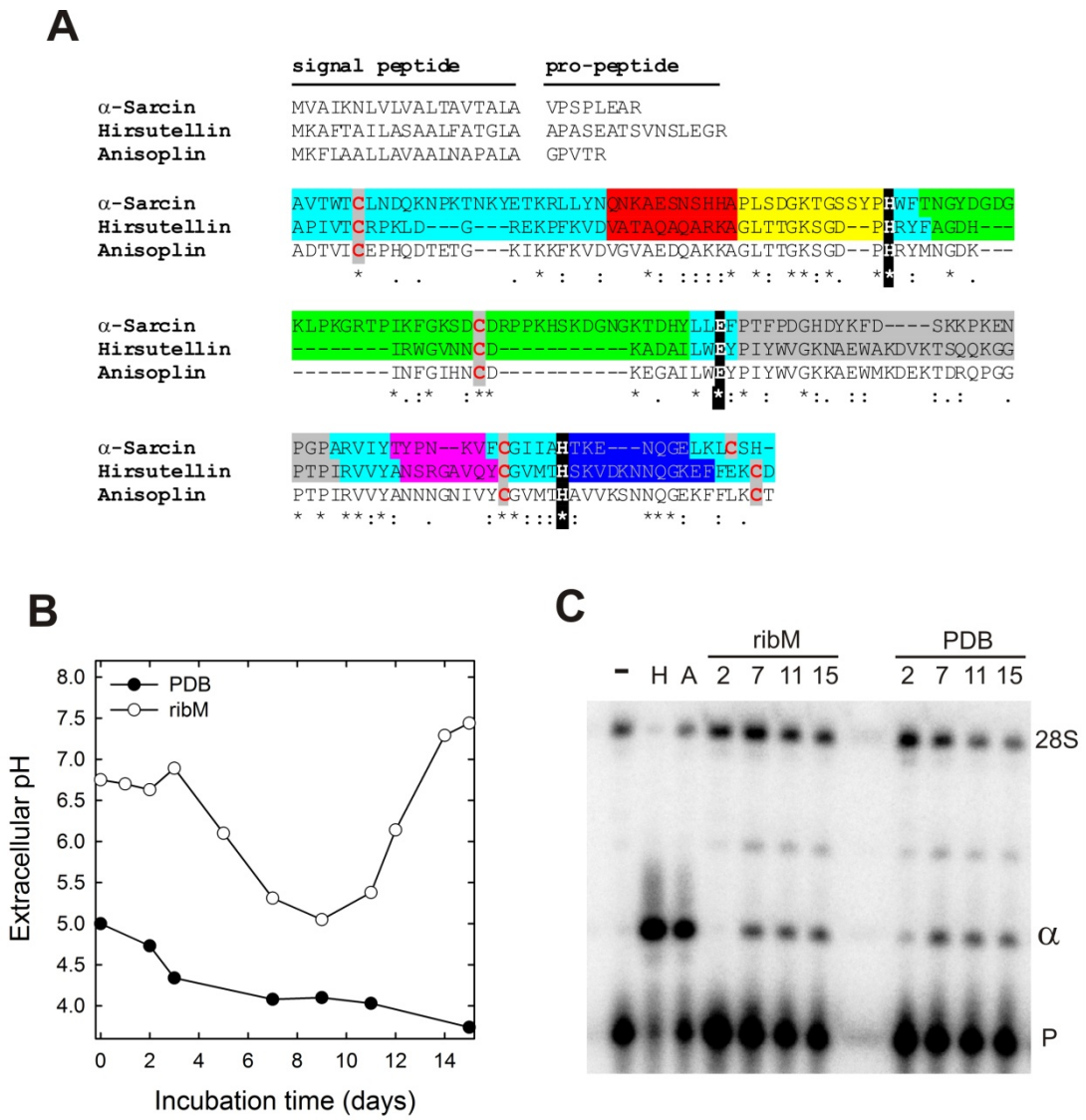


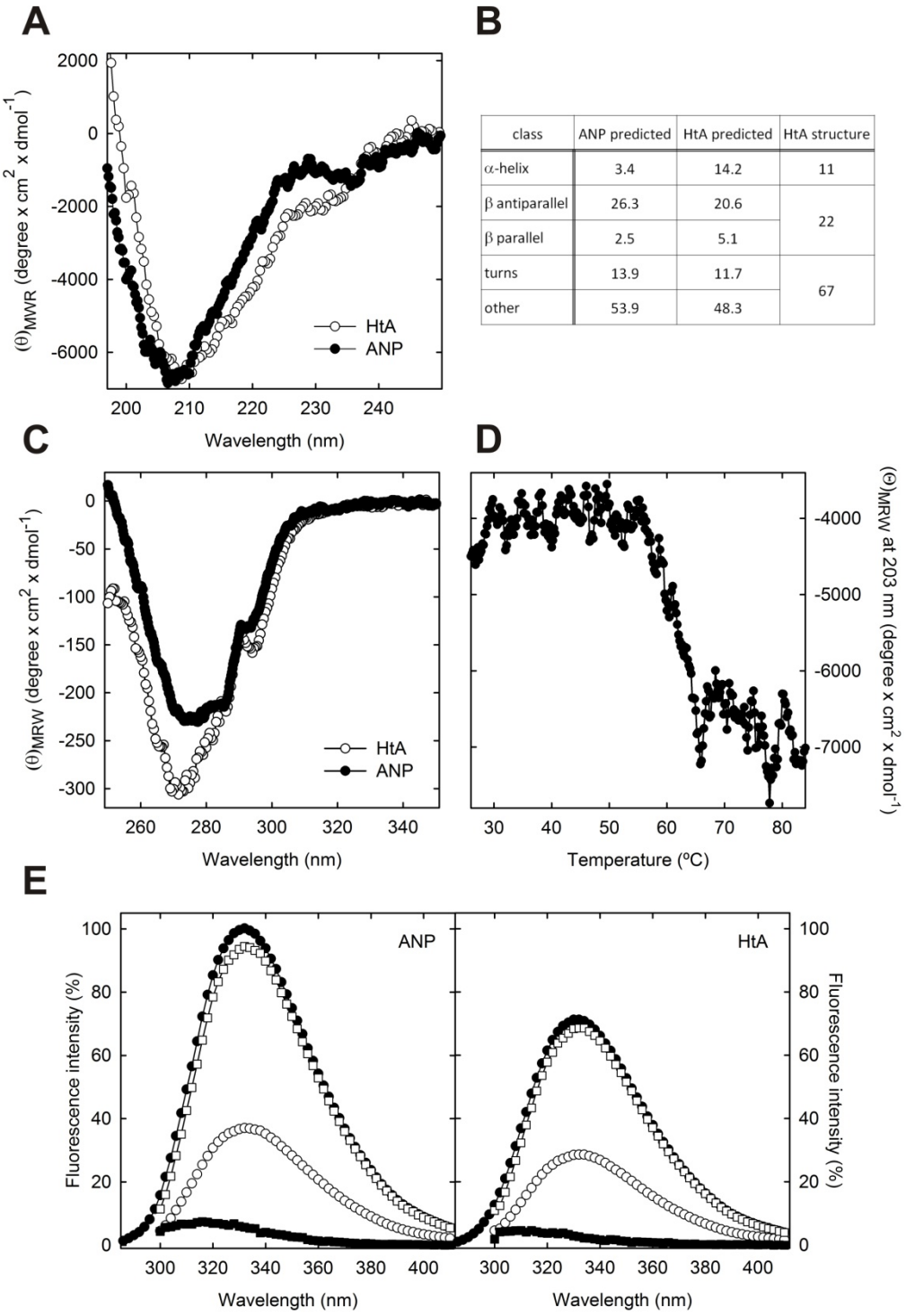
Figure 1.- A. Sequence alignment of anisoplin (accession number EFY94422.1), HtA and α-sarcin [13]. Colors indicate the secondary structure elements from the structures of HtA and α-sarcin: N-terminal β-hairpin and β strands in blue; α–helix in red and surrounding unstructured loops 1 to 5 in yellow, green, grey, pink and purple respectively. Catalytic residues are marked in black and cysteines in red/grey. Pre and pro peptides are indicated by comparison with those published for HtA and α-sarcin [26; 27]. **B.** Extracellular pH progression of *Metarhizium anisopliae* ARSEF23 in two different liquid media: PDB and ribotoxin production medium (ribM). **C.** Ribotoxin specific activity analysis of extracellular media from *M. anisopliae* cultures in B. Rabbit reticulocyte lysates were incubated with culture media, total RNA was extracted and ribotoxin specific cleavage was analyzed by poison primer extension. The band corresponding to the specific cleavage is marked with α. "P" corresponds to the free primer and "28S" to the resulted product of primer extension of intact 28S rRNA. Controls with purified fungal HtA (H), recombinant anisoplin (A) and in the absence of ribotoxin (-) are also included. Detailed methods are described in Supplementary material.

vitro conditions, mainly produces α-sarcin when culture is saturated and pH starts rising, correlating α-sarcin production to stress conditions[32]. Still, results with *M.*

anisopliae are not surprising considering that natural growth conditions of this fungus are completely different than those used in the laboratory. Natural sources of *M. anisopliae* are not submerged cultures. Instead, it is a very ubiquitous fungus from the soil. This is, however an interesting aspect to be explored in a near future: how and when, in its natural environment, *M. anisopliae* produces this ribotoxin in order to assign a biological function to it.

In order to carefully characterize this ribotoxin, it was recombinantly produced in *Escherichia coli* BL21(DE3) cells. The same expression system previously optimized for the production of α-sarcin was employed [33;34] (Supplementary material). It produces a fusion protein directed to the periplasm and the coexpression of tiorredoxin to favor disulphide bridges formation. Cloning the coding region of the anisoplin gene into the expression vector pINPGOmpA* renders a mature protein with an identical sequence than the fungal protein but an amino acid change in second position (Supplementary material). Protein purification followed the procedure described for the recombinant α-sarcin [33] yielding 0.5 mg of pure protein per liter of original culture. Protein size and homogeneity was checked by SDS-PAGE. Amino acid composition analysis agreed with the theoretical values and together with the absorbance spectrum allowed the calculation of the extinction coefficient: $E^{0.1\%}$ (280 nm, 1cm) = 1.62. Tryptic digestion and MALDI-TOF/TOF mass spectrometry identified the mature anisoplin with 60% sequence coverage.

The structural characterization of the recombinant anisoplin was performed by circular dichroism and intrinsic fluorescence emission spectroscopy. Far-UV CD spectrum showed slight differences in comparison with HtA[14] (Fig. 2A). Prediction of secondary structure content from the CD data was performed with *BeStSel* method [35]. Results were similar to those of HtA, corroborated with the real structure (PDB ID 2KAA) (Fig. 2B). Considering these data and the high sequence identity between anisoplin and HtA, the former seems to keep the same overall protein fold of ribotoxins, with a core of antiparalel β-sheet, a short α-helix and long unstructured loops surrounding it. In fact, modeling the structure with *Phyre*^{2} resulted in a similar protein to HtA, with 97% of the residues modeled at >90% confidence [36]. A closer look to both sequences highlights only remarkable differences in the NH_2-terminal β-hairpin, being three amino acids longer in anisoplin (Fig. 1A). It is possible that the β content of the Nt-hairpin accounts for the difference in far-UV CD spectra. Near-UV CD spectrum is unique for each protein since it depends on the tertiary structure relationships involving aromatic residues. The spectrum for anisoplin was however similar to that of HtA (Fig. 2C) [14], corresponding to that of a folded protein where the microenviroment of aromatic residues seem to be conserved. In fact, the five Tyr, three Trp and three of the four Phe of HtA are conserved in anisoplin. Anisoplin lacks Trp-52 and Phe-45, instead it has Phe-55. These differences and the presence of a proline contiguous to Phe-17 in HtA sequence could explain the differences in the near-UV CD spectra.



class	ANP predicted	HtA predicted	HtA structure
α-helix	3.4	14.2	11
β antiparallel	26.3	20.6	22
β parallel	2.5	5.1	
turns	13.9	11.7	67
other	53.9	48.3	

Figure 2.- Structural characterization of recombinant anisoplin (ANP) compared to hirsutellin A (HtA). **A**. Far-UV circular dichroism spectra. **B.** Secondary structure prediction from the spectra in A using *BestSel* online software [35]. Real data from HtA NMR structure are also included [50]. **C.** Near-UV circular dichroism spectra. **D.** Thermal denaturation profile of anisoplin by means of the temperature dependence of the ellipticity at 203 nm. Temperature continuously changed at 0.5 ºC/min. **E.** Fluorescence emission spectra of anisoplin and HtA for excitation at 275 nm (filled circles, total Tyrosine and Tryptophan contribution) and 295 nm (open circles). Open squares spectra correspond to Tyrosine contribution (after normalization of 295 nm spectra) and filled square spectra are Tryptophan contribution. Fluorescence units are arbitrary. All experiments were performed in 50 mM sodium phosphate pH 7.0, 0.1 M NaCl. Procedures followed those previously described [14] and are also detailed in Supplementary material.

Thermal denaturation profile was obtained by recording the ellipticity at 203 nm (wavelength were the maximum transition occurs) over the temperature (Fig. 2D). The curve corresponded to a folded-unfolded transition with a Tm (temperature at the midpoint of the observed single phase thermal transition) of 61ºC, similar to HtA, pointing out that the characterized recombinant anisoplin is a folded and fairly stable protein.

Finally, and in addition to the near-UV CD spectra, the fluorescence emission spectra for excitation at both 275 and 295 nm were obtained in comparison with HtA (Fig 2E and F) in order to analyze the structural microenvironment of Trp and Tyr residues. As it occurs with HtA, the total emission spectrum mainly corresponds to Trp contribution with almost negligible Tyr contribution. However, the Trp emission is significantly increased in anisoplin despite harboring three instead of four Trp. Taking these and the CD results together and considering that the sequence stretch harboring the three conserved Trp is almost identical between anisoplin and HtA (Fig 1A), the differences in fluorescence emission seem to respond to local differences in the microenviroment of Trp residues due to interactions with amino acids in close proximity in the structure although far in the sequence.

All together, the structural characterization of recombinant anisoplin shows a folded protein, similar in structure to its homologous HtA with subtle differences easily explained by the slight variation in amino acid sequences.

Recombinant anisoplin was next assayed in order to describe its behavior in standard experiments that show typical ribotoxin activity. As mentioned before, ribotoxins are extremely specific RNases against a unique element present in any kind of ribosome: the sarcin/ricin loop (SRL) [37;38]. This is a conserved sequence of about 35 nucleotides of the large ribosomal RNA essential for elongation factor action during protein biosynthesis [19;39;40;41]. This role of the SRL can be impaired by two types of toxins: N-glycosidades like ricin, best known as RIPs (ribosome inactivating proteins), that depurinate one nucleotide [42] and ribotoxins, that hydrolyze the contiguous phosphodiester bond [13;17]. These modifications in the SRL, even when do not cause the loss of structure, completely inactivate the mechanisms of correct positioning and GTPase activation of elongation factors that it is involved in, leading to protein biosynthesis inactivation and finally cell death.

Ribotoxins specifically recognize the position of the phosphodiester bond to cleave, not only within the complete ribosome, but also with an isolated SRL analogous as substrate. However, ribotoxins action over the ribosome is much more efficient. In this regard, several studies have tried to specify the structural elements in the ribotoxin, the ribosome and the SRL responsible for this fine and efficient recognition [20;43;44].

Here, recombinant anisoplin was first tested against a 35-mer SRL analogous according to previously described methods and in comparison with HtA [14;43] (Fig. 3A and B). Anisoplin specifically cleaved the SRL mimic yielding two fragments of 21 and

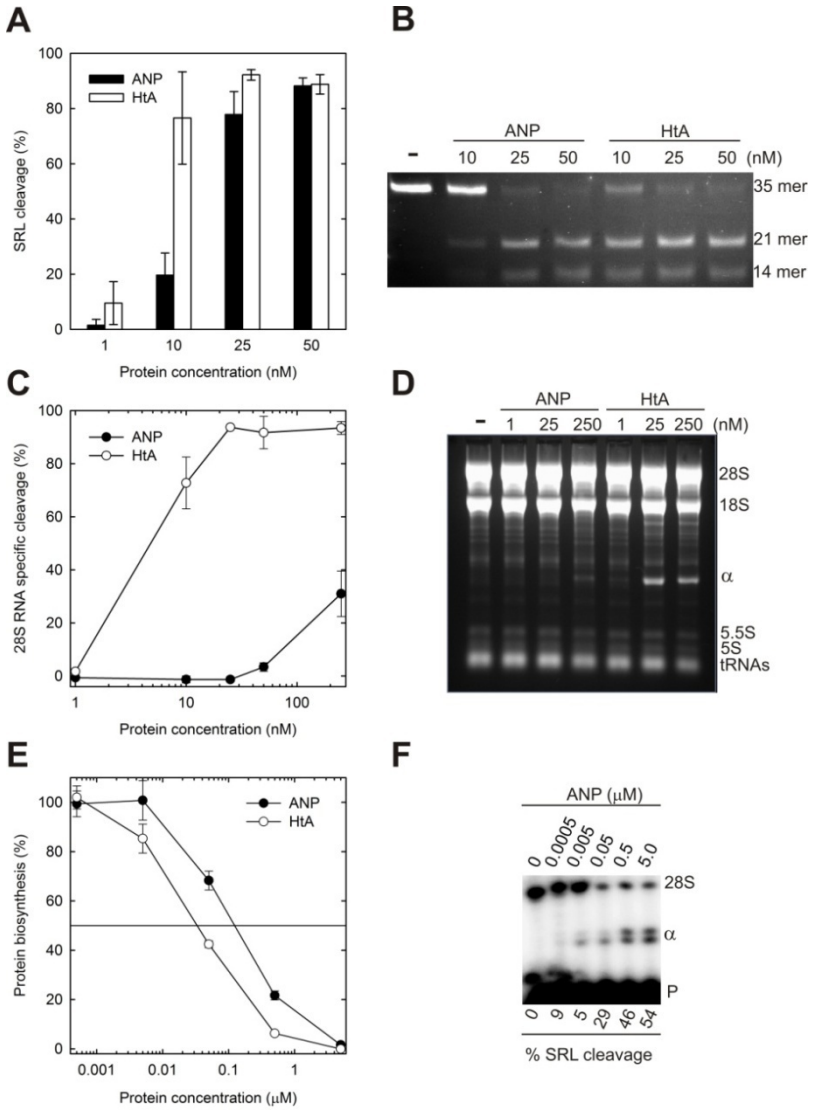


Figure 3.- Ribonucleolytic and cytotoxic activities of recombinant anisoplin (ANP) compared to hirsutellin A (HtA). **A.** Quantitation of specific activity against a 35-mer sarcin/ricin loop analogous. SRL-like RNA was *in vitro* transcribed and gel purified. 2 μM substrate was assayed against different concentrations of protein for 15 min at 37ºC in 50 mM Tris-HCl pH 7.0 and 1 mM $MgCl_2$. **B**. Analysis of the SRL cleavage by denaturing electrophoresis separation of products and ethidium bromide staining. Sizes of substrate and products resulted from the specific ribotoxin cleavage of one single phosphodiester bond are indicated. **C.** Quantitation of specific ribonucleolytic activity of ribotoxins against a rabbit reticulocyte lysate (Promega). Reactions with different concentrations of proteins were performed for 15 min at 25ºC in the presence of lysate diluted in 25 mM Tris-HCl, 25 mM KCl, 6 mM EDTA pH 7.5. **D.** Analysis of the specific cleavage of 28S rRNA in reticulocytes lysates by denaturing electrophoresis and ethidium bromide staining. Bands corresponding to the different ribosomal RNAs and the specific α-fragment (α) released by the ribotoxin are indicated. **E.** Protein biosynthesis inhibition by ribotoxins on Sf9 insect cells. Monolayer cultures were incubated with different concentrations of ribotoxins for 18 hours at 27ºC. Protein biosynthesis was quantitated by the incorporation of ^{3}H-Leu to newly synthesized proteins. **F.** Poison primer extension analysis of RNA extracted from Sf9 cells treated with anisoplin as in E. Bands corresponding to the ribotoxin specific cleavage (α), free primer (P) and not cleaved 28S are indicated as well as protein concentrations and percentage of cleavage. All results are expressed as the average of at least three independent experiments ± SD. Procedures followed those previously described [14; 16; 43], and are detailed in Supplementary material.

14 mer which corresponds to the typical pattern shown by ribotoxins. Moreover, this specific cleavage activity saturates at the same protein concentration as it does with HtA. So we can say that anisoplin recognizes and cleaves the SRL as HtA does. Ribosomes were next analyzed as substrates of anisoplin by assaying it and HtA against a rabbit reticulocyte lisate as previously described [14;43] (Fig. 3C and D). In this assay, the specific cleavage of the 18S RNA within the ribosomes by the ribotoxin releases a RNA fragment of about 400 nucleotides, the so-called α-fragment. Again, anisoplin behaves as a ribotoxin, being able to release the α-fragment. However, when compared with HtA, it is considerably less active in terms of protein concentration requirement to reach total RNA specific cleavage (more than 50 times). Therefore ribosome recognition seems to be different in these two proteins, at least in terms of efficiency. Important differences in this regard have also been observed in our laboratory between α-sarcin and HtA [14;16] although they are more striking in this case, being HtA and anisoplin such similar proteins in terms of sequence identity. Further characterization of ribosome recognition by these two ribotoxins will shed light into the molecular mechanism of this process, something very interesting considering the high efficiency and specificity of ribotoxins. To date, the only structural element of ribotoxins involved in ribosome recognition that has been described is the Nt β-hairpin [43;44;45]. Others like loop 2 have been suggested to participate in the process [44]. Interestingly, a close examination of α-sarcin, HtA and anisoplin sequences reveals major differences in these two elements (Fig. 1A). Loop 2 is much shorter in HtA and anisoplin than it is in α-sarcin and the rest of ribotoxins known [13]. More importantly, the Nt β-hairpin is also shorter in HtA and anisoplin than it is in α-sarcin but here, differences between HtA and anisoplin are more dramatic, as it has been commented above. Anisoplin β-hairpin is longer, with a theoretical pI of 5.5 instead of 9.2 for HtA. This different Nt β-hairpin could be responsible for the differences in activity against ribosomes observed between anisoplin and HtA, something worth to be deeply explored.

Finally, ribotoxins are cytotoxic against a variety of cells thanks to their ability to cross lipid membranes previous to their inactivating function over the ribosomes. The specificity here, since they do not interact with any membrane receptor, is the lipid composition of the membrane of the target cell. Recently, it has been shown that ribotoxins display a much higher toxicity against insect than mammalian transformed cells and their insecticidal properties have been described [16]. A similar experiment of protein biosynthesis inhibition with Sf9 insect cells has been performed here (Fig. 3E). Anisoplin was able to inhibit protein biosynthesis with an IC_{50} of 120 nM, similar to HtA and α-sarcin and in good agreement with the effect of these three ribotoxins against ribosomes of a rabbit reticulocyte lysate (Fig 3C and D)[16]. Moreover, in order to correlate this effect with ribosome inhibition, RNA from Sf9 cell treated with anisoplin was extracted and analyzed by primer extension (Fig. 3F). Results showed the specific fragment due to ribotoxin cleavage in percentages similar to those obtained with HtA

and α-sarcin [16]. Therefore, anisoplin, as a tipical ribotoxin, causes cytotoxicity in cultured insect cells by specifically cleaving their ribosomes.

In this report we describe anisoplin, a ribotoxin from the entomopathogenic fungus *Metarhizium anisopliae*. The characteristic ribotoxin cleavage activity has been observed in extracellular extracts of *M. anisopliae*. However, it is still not known how and when this toxin is produced by the fungus in nature. Its toxic character suggests some relevant function during insect infection but further research needs to be done. Other ribotoxins like restrictocin have a deterrent effect on insects feeding as a protection mechanism during conidia maturation of *Aspergillus* [18;46]. But for the closest related, HtA, there is poor correlation between its production and the toxicity exerted by the fungus [47]. Considering the importance of *Metarhizium* in a broad spectrum of biotechnological applications, including malaria vector control, the characterization of its toxic metabolites and secreted proteins involved in pathogenesis is a useful tool [1;48;49]. Even efforts have been recently made in order to predict, characterize and compare the secretome of diverse entomopathogenic fungi [48;49]. However, there was not previous evidence of ribotoxins in *Metarhizium* apart from the results obtained from various sequenced genomes. In fact, not only *M. anisopliae* but also *M. roberstii* and *M. acridum,* with different insect specificities, have shown sequences almost identical to anisoplin in their genomes (accession numbers KFG678708.1, EFY94422.1 and EFY89817.1) [8;49]. In this regard, the characterization herein performed shows anisoplin as a protein highly cytotoxic to insect cells with interesting features when compared with other fungal ribotoxins in terms of its structure, in particular its unique Nt β-hairpin, as well as its ribonucleolytic activity. Moreover, fungal ribotoxins have been recently described as insecticidal agents [16]. With all this, the ribotoxin presented here, anisoplin, seems to be an interesting biological weapon of *Metarhizium.* Its potential as a biotechnological tool in agricultural pest and disease-vector control encourages further studies regarding its molecular features as a ribotoxin as well as its insecticide properties not to forget its role in nature.

References

1. Schrank, A. & Vainstein, M. H. (2010). *Metarhizium anisopliae* enzymes and toxins. *Toxicon* **56**, 1267-74.
2. Faria, M. R. d. & Wraight, S. P. (2007). Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control* **43**, 237-256.
3. Kanga, L. H., James, R. R. & Boucias, D. G. (2002). *Hirsutella thompsonii* and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor*, a honey bee parasite. *J Invertebr Pathol* **81**, 175-84.
4. Kanzok, S. M. & Jacobs-Lorena, M. (2006). Entomopathogenic fungi as biological insecticides to control malaria. *Trends Parasitol* **22**, 49-51.
5. Mnyone, L. L., Kirby, M. J., Lwetoijera, D. W., Mpingwa, M. W., Knols, B. G., Takken, W. & Russell, T. L. (2009). Infection of the malaria mosquito, *Anopheles gambiae*, with two species of

entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence. *Malar J* **8**, 309.

6. Howard, A. F., Koenraadt, C. J., Farenhorst, M., Knols, B. G. & Takken, W. (2010). Pyrethroid resistance in *Anopheles gambiae* leads to increased susceptibility to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Malar J* **9**, 168.
7. Abdul-Ghani, R., Al-Mekhlafi, A. M. & Alabsi, M. S. (2012). Microbial control of malaria: biological warfare against the parasite and its vector. *Acta Trop* **121**, 71-84.
8. Gao, Q., Jin, K., Ying, S. H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X. Q., Zhou, G., Peng, G., Luo, Z., Huang, W., Wang, B., Fang, W., Wang, S., Zhong, Y., Ma, L. J., St Leger, R. J., Zhao, G. P., Pei, Y., Feng, M. G., Xia, Y. & Wang, C. (2011). Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genet* **7**, e1001264.
9. Pattemore, J. A., Hane, J. K., Williams, A. H., Wilson, B. A., Stodart, B. J. & Ash, G. J. (2014). The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. *BMC Genomics* **15**, 660.
10. Wang, B., Kang, Q., Lu, Y., Bai, L. & Wang, C. (2012). Unveiling the biosynthetic puzzle of destruxins in *Metarhizium* species. *Proc Natl Acad Sci U S A* **109**, 1287-92.
11. Scholte, E. J., Knols, B. G., Samson, R. A. & Takken, W. (2004). Entomopathogenic fungi for mosquito control: a review. *J Insect Sci* **4**, 19.
12. Ortiz-Urquiza, A., Luo, Z. & Keyhani, N. O. (2015). Improving mycoinsecticides for insect biological control. *Appl Microbiol Biotechnol* **99**, 1057-68.
13. Lacadena, J., Álvarez-García, E., Carreras-Sangrá, N., Herrero-Galán, E., Alegre-Cebollada, J., García-Ortega, L., Oñaderra, M., Gavilanes, J. G. & Martínez del Pozo, A. (2007). Fungal ribotoxins: molecular dissection of a family of natural killers. *FEMS Microbiol Rev* **31**, 212-37.
14. Herrero-Galán, E., Lacadena, J., Martínez del Pozo, A., Boucias, D. G., Olmo, N., Oñaderra, M. & Gavilanes, J. G. (2008). The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. *Proteins* **72**, 217-28.
15. Martínez-Ruiz, A., Martínez del Pozo, A., Lacadena, J., Oñaderra, M. & Gavilanes, J. G. (1999). Hirsutellin A displays significant homology to microbial extracellular ribonucleases. *J Invertebr Pathol* **74**, 96-7.
16. Olombrada, M., Herrero-Galán, E., Tello, D., Oñaderra, M., Gavilanes, J. G., Martínez-del-Pozo, A. & García-Ortega, L. (2013). Fungal extracellular ribotoxins as insecticidal agents. *Insect Biochem Mol Biol* **43**, 39-46.
17. Olombrada, M., Martínez-del-Pozo, A., Medina, P., Budia, F., Gavilanes, J. G. & García-Ortega, L. (2014). Fungal ribotoxins: Natural protein-based weapons against insects. *Toxicon* **83**, 69-74.
18. Brandhorst, T. T. & Kenealy, W. R. (1992). Production and localization of restrictocin in *Aspergillus restrictus*. *J Gen Microbiol* **138**, 1429-35.
19. García-Ortega, L., Álvarez-García, E., Gavilanes, J. G., Martínez-del-Pozo, A. & Joseph, S. (2010). Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. *Nucleic Acids Res* **38**, 4108-19.
20. Olombrada, M., Rodríguez-Mateos, M., Prieto, D., Pla, J., Remacha, M., Martínez-del-Pozo, A., Gavilanes, J. G., Ballesta, J. P. & García-Ortega, L. (2014). The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by alpha-sarcin of the eukaryotic ribosome. *Biochemistry* **53**, 1545-7.
21. Carreras-Sangrá, N., Tomé-Amat, J., García-Ortega, L., Batt, C. A., Oñaderra, M., Martínez-del-Pozo, A., Gavilanes, J. G. & Lacadena, J. (2012). Production and characterization of a colon cancer-specific immunotoxin based on the fungal ribotoxin alpha-sarcin. *Protein Eng Des Sel* **25**, 425-35.
22. Tomé-Amat, J., Olombrada, M., Ruiz-de-la-Herrán, J., Pérez-Gómez, E., Andradas, C., Sanchez, C., Martínez, L., Martínez-Del-Pozo, A., Gavilanes, J. G. & Lacadena, J. (2015). Efficient in vivo antitumor

effect of an immunotoxin based on ribotoxin alpha-sarcin in nude mice bearing human colorectal cancer xenografts. *Springerplus* **4**, 168.

23. Oka, T., Natori, Y., Tanaka, S., Tsurugi, K. & Endo, Y. (1990). Complete nucleotide sequence of cDNA for the cytotoxin alpha sarcin. *Nucleic Acids Res* **18**, 1897.
24. Endo, Y., Oka, T., Tsurugi, K. & Natori, Y. (1993). The biosynthesis of a cytotoxic protein, alpha-sarcin, in a mold *Aspergillus giganteus*. I. Synthesis of prepro- and pro-alpha-sarcin in vitro. *Tokushima J Exp Med* **40**, 1-6.
25. Endo, Y., Oka, T., Yokota, S., Tsurugi, K. & Natori, Y. (1993). The biosynthesis of a cytotoxic protein, alpha-sarcin, in a mold of *Aspergillus giganteus*. II. Maturation of precursor form of alpha-sarcin in vivo. *Tokushima J Exp Med* **40**, 7-12.
26. Boucias, D. G., Farmerie, W. G. & Pendland, J. C. (1998). Cloning and sequencing of cDNA of the insecticidal toxin hirsutellin A. *J Invertebr Pathol* **72**, 258-61.
27. Martínez-Ruiz, A., Martínez del Pozo, A., Lacadena, J., Mancheño, J. M., Oñaderra, M., Lopez-Otín, C. & Gavilanes, J. G. (1998). Secretion of recombinant pro- and mature fungal alpha-sarcin ribotoxin by the methylotrophic yeast *Pichia pastoris*: the Lys-Arg motif is required for maturation. *Protein Expr Purif* **12**, 315-22.
28. Lai, Y., Liu, K., Zhang, X., Zhang, X., Li, K., Wang, N., Shu, C., Wu, Y., Wang, C., Bushley, K. E., Xiang, M. & Liu, X. (2014). Comparative genomics and transcriptomics analyses reveal divergent lifestyle features of nematode endoparasitic fungus *Hirsutella minnesotensis*. *Genome Biol Evol* **6**, 3077-93.
29. Lamy, B. & Davies, J. (1991). Isolation and nucleotide sequence of the *Aspergillus restrictus* gene coding for the ribonucleolytic toxin restrictocin and its expression in *Aspergillus nidulans*: the leader sequence protects producing strains from suicide. *Nucleic Acids Res* **19**, 1001-6.
30. Chen, H. (2012). Exploiting the Intron-splicing Mechanism of Insect Cells to Produce Viral Vectors Harboring Toxic Genes for Suicide Gene Therapy. *Mol Ther Nucleic Acids* **1**, e57.
31. Mazet, I. & Vey, A. (1995). Hirsutellin A, a toxic protein produced in vitro by *Hirsutella thompsonii*. *Microbiology* **141 (Pt 6)**, 1343-8.
32. Olson, B. H., Jennings, J. C., Roga, V., Junek, A. J. & Schuurmans, D. M. (1965). Alpha Sarcin, a New Antitumor Agent: II. Fermentation and Antitumor Spectrum. *Appl Microbiol* **13**, 322-6.
33. Lacadena, J., Martínez del Pozo, A., Barbero, J. L., Mancheño, J. M., Gasset, M., Oñaderra, M., Lopez-Otín, C., Ortega, S., García, J. & Gavilanes, J. G. (1994). Overproduction and purification of biologically active native fungal alpha-sarcin in *Escherichia coli*. *Gene* **142**, 147-51.
34. García-Ortega, L., Lacadena, J., Lacadena, V., Masip, M., De Antonio, C., Martínez-Ruiz, A. & Martínez Del Pozo, A. (2000). The solubility of the ribotoxin alpha-sarcin, produced as a recombinant protein in *Escherichia coli*, is increased in the presence of thioredoxin. *Lett Appl Microbiol* **30**, 298-302.
35. Micsonai, A., Wien, F., Kernya, L., Lee, Y. H., Goto, Y., Refregiers, M. & Kardos, J. (2015). Accurate secondary structure prediction and fold recognition for circular dichroism spectroscopy. *Proc Natl Acad Sci U S A* **112**, E3095-103.
36. Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. & Sternberg, M. J. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* **10**, 845-58.
37. Schindler, D. G. & Davies, J. E. (1977). Specific cleavage of ribosomal RNA caused by alpha sarcin. *Nucleic Acids Res* **4**, 1097-1110.
38. Endo, Y. & Wool, I. G. (1982). The site of action of alpha-sarcin on eukaryotic ribosomes. The sequence at the alpha-sarcin cleavage site in 28 S ribosomal ribonucleic acid. *J Biol Chem* **257**, 9054-60.
39. Voorhees, R. M., Schmeing, T. M., Kelley, A. C. & Ramakrishnan, V. (2010). The mechanism for activation of GTP hydrolysis on the ribosome. *Science* **330**, 835-8.
40. Shi, X., Khade, P. K., Sanbonmatsu, K. Y. & Joseph, S. (2012). Functional role of the sarcin-ricin loop of the 23S rRNA in the elongation cycle of protein synthesis. *J Mol Biol* **419**, 125-38.

41. Koch, M., Flur, S., Kreutz, C., Ennifar, E., Micura, R. & Polacek, N. (2015). Role of a ribosomal RNA phosphate oxygen during the EF-G-triggered GTP hydrolysis. *Proc Natl Acad Sci U S A* **112**, E2561-8.
42. Stirpe, F. & Battelli, M. G. (2006). Ribosome-inactivating proteins: progress and problems. *Cell Mol Life Sci* **63**, 1850-66.
43. García-Ortega, L., Masip, M., Mancheño, J. M., Oñaderra, M., Lizarbe, M. A., García-Mayoral, M. F., Bruix, M., Martínez del Pozo, A. & Gavilanes, J. G. (2002). Deletion of the NH2-terminal beta-hairpin of the ribotoxin alpha-sarcin produces a nontoxic but active ribonuclease. *J Biol Chem* **277**, 18632-9.
44. García-Mayoral, F., García-Ortega, L., Álvarez-García, E., Bruix, M., Gavilanes, J. G. & del Pozo, A. M. (2005). Modeling the highly specific ribotoxin recognition of ribosomes. *FEBS Lett* **579**, 6859-64.
45. Álvarez-García, E., Martínez-del-Pozo, A. & Gavilanes, J. G. (2009). Role of the basic character of alpha-sarcin's NH2-terminal beta-hairpin in ribosome recognition and phospholipid interaction. *Arch Biochem Biophys* **481**, 37-44.
46. Brandhorst, T., Dowd, P. F. & Kenealy, W. R. (1996). The ribosome-inactivating protein restrictocin deters insect feeding on *Aspergillus restrictus*. *Microbiology* **142 (Pt 6)**, 1551-6.
47. Maimala, S., Tartar, A., Boucias, D. & Chandrapatya, A. (2002). Detection of the toxin Hirsutellin A from *Hirsutella thompsonii*. *J Invertebr Pathol* **80**, 112-26.
48. de Bekker, C., Smith, P. B., Patterson, A. D. & Hughes, D. P. (2013). Metabolomics reveals the heterogeneous secretome of two entomopathogenic fungi to ex vivo cultured insect tissues. *PLoS One* **8**, e70609.
49. Staats, C. C., Junges, A., Guedes, R. L., Thompson, C. E., de Morais, G. L., Boldo, J. T., de Almeida, L. G., Andreis, F. C., Gerber, A. L., Sbaraini, N., da Paixao, R. L., Broetto, L., Landell, M., Santi, L., Beys-da-Silva, W. O., Silveira, C. P., Serrano, T. R., de Oliveira, E. S., Kmetzsch, L., Vainstein, M. H., de Vasconcelos, A. T. & Schrank, A. (2014). Comparative genome analysis of entomopathogenic fungi reveals a complex set of secreted proteins. *BMC Genomics* **15**, 822.
50. Viegas, A., Herrero-Galán, E., Oñaderra, M., Macedo, A. L. & Bruix, M. (2009). Solution structure of hirsutellin A--new insights into the active site and interacting interfaces of ribotoxins. *FEBS J* **276**, 2381-90.

Supplementary Material: Experimental Procedures

Fungal cultures

Metarhizium anisopliae ARSEF 23 was obtained from ATCC (ATCC® MYA-3075™) and it was maintained in PDA plates. Liquid cultures were started in 20 mL of standard PDB or the ribotoxin production medium as described [1] (2% peptone, 2% corn starch, 0.5% NaCl and 1.5%), and after 4 days of growth (25ºC, 100 rpm) 5 mL were transferred to 75 mL of medium. Cultures were maintained in the same growth conditions for 15 days. Aliquots were daily extracted for pH measurements and ribotoxin activity analyses of the extracellular media.

Anisoplin cloning, expression and purification

Anisoplin gene contains an intron between 2nd and 3rd codons of the mature protein (accession number MAA_10099). Therefore cloning was possible with total DNA from *M. anisopliae* as template and using the following primers for PCR amplification:

5'-CTAACAGCGGTGACCGTTATTTGCGAACCTCAC-3' (xxx-BstEII/AVT-VICEPH) and
5'-ACTAAGCCGGATCCAGATCAGGTGCACTTCAGGAA-3' (xxx-BamHI-StopTCKLF).

In this way anisoplin cDNA could be easily inserted into the pINPGOmpA* vector [2;3] just by changing D by V amino acid in second position. All cloning procedures and enzymatic reactions were carried out following standard procedures [4]. Anisoplin insert was verified by sequence analysis at the Universidad Complutense facility. Recombinant anisoplin was produced in *Escherichia coli* BL21(DE3) cells harboring pINPGOmpA-ANP and pT-Tx plasmids by growing cells in LB media and inducing with IPTG. Production and purification followed the procedure described for recombinant α-sarcin [2;3]. Protein homogeneity was checked by polyacrilamide gel electrophoresis followed by Coomassie blue staining and amino acid analysis according to standard procedures [3]. Fungal hirsutellin A was purified from *Hirsutella thompsonii var. thompsonii* HTF72 as described before [5].

Structural characterization

Spectroscopic characterization was performed following well-established procedures [6;7;8;9;10;11;12]. Absorbance measurements were carried out on a Beckman DU640 spectrophotometer (Beckman Coulter, Brea, CA, USA) at 100 nm/min scanning speed and room temperature. Amino acid analysis and the UV-absorbance spectrum were also used to calculate the extinction coefficient of anisoplin. Circular dichroism spectra were obtained in a Jasco 715 spectropolarimeter (Jasco, Easton, MD, USA), equipped with a thermostated cell holder and a Neslab-111 circulating water bath, at 0.2 nm/s scanning speed. 0.1 cm optical path cell and about 0.1 mg/mL protein concentration were employed for far-UV CD spectra For near-UV CD spectra, 1 cm optical path cell and 0.5 mg/mL protein concentration were required. Thermal

denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 203 nm in the 25–80°C range using a constant rate of temperature increment of 30°C per hour. Fluorescence emission spectra were recorded on an SLM Aminco 8000 spectrofluorimeter at 25°C using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission beams, respectively, were used and protein concentration was 0.05 mg/mL. All these experiments were performed in 50 mM sodium phosphate, pH 7.0, containing 0.1 M NaCl.

Mass spectrometry analysis

This analysis was performed by the Proteomic Unit of CIB-CSIC, Madrid, Spain. Briefly, recombinant pure anisoplin was separated in a SDS-PAGE and the protein from the gel piece was extracted and subjected to tryptic digestion. Peptide mixtures were analyzed with an Autoflex III TOF/TOF mass spectrometer (Bruker-Daltonics).Typically, 1000 scans for peptide mass fingerprinting (PMF) and 2000 scans for MS/MS were collected. Automated analysis of mass data was performed using FlexAnalysis software (Bruker-Daltonics). MALDI–MS and MS/MS data were combined through the BioTools 3.0 program (Bruker-Daltonics) to interrogate the NCBI non-redundant protein database (NCBI: 20140323) using MASCOT software 2.3 (Matrix Science).

Ribonucleolytic activity assays

All procedures were carried out using RNase-free materials and reagents. The specific ribonucleolytic activity of ribotoxins is usually detected by the release of a 400-nt fragment (*α-fragment*) from eukaryotic ribosomes [13]. Therefore, different concentrations of anisoplin, hirsutellin A (from 1 to 250 nM) and extracellular media from *M. anisopliae* cultures were assayed against ribosomes contained in a Promega rabbit cell-free reticulocyte lysate [14]. After a 15 min incubation at 25ºC in 23 mM Tris-HCl, 23 mM KCl, 6 mM EDTA pH 7.5, RNA was phenol-extracted, precipitated with isopropanol and visualized by ethidium bromide staining after electrophoresis on denaturing 2.4 % agarose gels as described [3;5;11]. Band intensities were quantitated with QuantityOne Software and percentage of specific cleavage was expressed as: α/18S, considering 100% the α-fragment produced by 250 nM HtA.

Ribotoxin specific cleavage was also quantitated by poison primer extension (PPE) as described [15;16]. This method was specially employed when insect ribosomes were assayed or in those cases with very low ribotoxin activity. Reverse transcription where dATP is substituted by ddATP was performed using the complementary sequence downstream the SRL in the 28S rRNA of *O. cuniculus* (5'-ACCAAATGTCTGAACCTGCGG-3'). The products of this reverse transcription were then separated in a denaturing 10 % polyacrylamide gel and the amount of ^{32}P present in

each one of the DNA bands produced was quantitated using a PhosphorImager screen (Molecular Dynamics). Percentage of specific cleavage was expressed as 100xα/(α+28S).

Finally, cleavage of a synthetic oligonucleotide that mimics the sequence and structure of the SRL was also measured for anisoplin and HtA. Synthesis of this SRL-like 35mer RNA was performed as previously described [14;15]. SRL (2 μM) was incubated with 1-50 nM protein for 15 min at 37°C in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM $MgCl_2$. Reaction products were run on a denaturing 19 % (w/v) polyacrylamide gel and visualized by ethidium bromide staining. Band intensities were quantitated with Quantity One Software and percentage of specific cleavage was expressed as: 100x(intact 35mer – 35mer)/intact 35mer.

Insect cell culture and toxicity assays

The insect cell line *Spodoptera frugiperda* (Sf9) was cultured at 27°C as described [17;18] in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker) as indicated by the manufacturer. Protein solutions were prepared in culture medium and sterilized by ultrafiltration. Protein biosynthesis assays were carried out by seeding Sf9 cells into 24-well plates at a cell density of 10^5 cells per well, maintaining them under standard culture conditions up to 80% confluence. Then, monolayer cultures were incubated in 0.5 mL of fresh medium with serial dilutions of ribotoxin from 5.0 μM to 0.5 nM final concentrations. Following 18 h of incubation at 27°C medium was replaced by culture medium supplemented with 0.5 μCi/well of [^{3}H]-leucine. After 5 h of incubation medium was removed and cell protein content was precipitated with 5% trichloroacetic acid and washed three times with ethanol. The precipitate was dried, dissolved in 200 μL of 0.1 N NaOH, 0.1% SDS and radioactivity was measured in a Beckman LS 3801 liquid scintillation counter. Results are expressed as percentage of incorporated radioactivity relative to samples without ribotoxin added.

Analysis of ribotoxin specific cleavage of ribosomes in Sf9 cells after treatment with anisoplin was also performed as described [18]. Therefore, cells were recovered in 200 μL of 100 mM HEPES-HCl pH 7.5, pelleted and resuspended in 200 μL QIAzol lysis reagent (Qiagen). RNA was then extracted from the aqueous phase, precipitated and resuspended in 10 μL H_2O. RNA specific cleavage was visualized as described above.

Supplementary references

1. Olson, B. H. & Goerner, G. L. (1965). ALPHA SARCIN, A NEW ANTITUMOR AGENT. I. ISOLATION, PURIFICATION, CHEMICAL COMPOSITION, AND THE IDENTITY OF A NEW AMINO ACID. *Appl Microbiol* **13**, 314-21.
2. García-Ortega, L., Lacadena, J., Lacadena, V., Masip, M., De Antonio, C., Martínez-Ruiz, A. & Martínez Del Pozo, A. (2000). The solubility of the ribotoxin alpha-sarcin, produced as a recombinant protein in *Escherichia coli*, is increased in the presence of thioredoxin. *Lett Appl Microbiol* **30**, 298-302.
3. Lacadena, J., Martínez del Pozo, A., Barbero, J. L., Mancheño, J. M., Gasset, M., Oñaderra, M., López-Otín, C., Ortega, S., García, J. & Gavilanes, J. G. (1994). Overproduction and purification of biologically active native fungal alpha-sarcin in *Escherichia coli*. *Gene* **142**, 147-51.

4. Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.
5. Herrero-Galán, E., Lacadena, J., Martínez del Pozo, A., Boucias, D. G., Olmo, N., Oñaderra, M. & Gavilanes, J. G. (2008). The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. *Proteins* **72**, 217-28.
6. Álvarez-García, E., García-Ortega, L., Verdun, Y., Bruix, M., Martínez del Pozo, A. & Gavilanes, J. G. (2006). Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of alpha-sarcin. *Biol Chem* **387**, 535-41.
7. Álvarez-García, E., Martínez-del-Pozo, A. & Gavilanes, J. G. (2009). Role of the basic character of alpha-sarcin's NH2-terminal beta-hairpin in ribosome recognition and phospholipid interaction. *Arch Biochem Biophys* **481**, 37-44.
8. García-Ortega, L., Lacadena, J., Villalba, M., Rodríguez, R., Crespo, J. F., Rodríguez, J., Pascual, C., Olmo, N., Oñaderra, M., del Pozo, A. M. & Gavilanes, J. G. (2005). Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Aspf1 displaying reduced IgE binding. *FEBS J* **272**, 2536-44.
9. García-Ortega, L., Masip, M., Mancheño, J. M., Oñaderra, M., Lizarbe, M. A., García-Mayoral, M. F., Bruix, M., Martínez del Pozo, A. & Gavilanes, J. G. (2002). Deletion of the NH2-terminal beta-hairpin of the ribotoxin alpha-sarcin produces a nontoxic but active ribonuclease. *J Biol Chem* **277**, 18632-9.
10. García-Ortega, L., Lacadena, J., Mancheño, J. M., Oñaderra, M., Kao, R., Davies, J., Olmo, N., Pozo, A. M. & Gavilanes, J. G. (2001). Involvement of the amino-terminal beta-hairpin of the *Aspergillus* ribotoxins on the interaction with membranes and nonspecific ribonuclease activity. *Protein Sci* **10**, 1658-68.
11. Lacadena, J., Martínez del Pozo, A., Martínez-Ruiz, A., Perez-Cañadillas, J. M., Bruix, M., Mancheño, J. M., Oñaderra, M. & Gavilanes, J. G. (1999). Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin alpha-sarcin. *Proteins* **37**, 474-84.
12. Martínez-Ruiz, A., García-Ortega, L., Kao, R., Lacadena, J., Oñaderra, M., Mancheño, J. M., Davies, J., Martinez del Pozo, A. & Gavilanes, J. G. (2001). RNase U2 and alpha-sarcin: a study of relationships. *Methods Enzymol* **341**, 335-51.
13. Schindler, D. G. & Davies, J. E. (1977). Specific cleavage of ribosomal RNA caused by alpha sarcin. *Nucleic Acids Res* **4**, 1097-1110.
14. Kao, R., Martínez-Ruiz, A., Martínez del Pozo, A., Crameri, R. & Davies, J. (2001). Mitogillin and related fungal ribotoxins. *Methods Enzymol* **341**, 324-35.
15. García-Ortega, L., Álvarez-García, E., Gavilanes, J. G., Martínez-del-Pozo, A. & Joseph, S. (2010). Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. *Nucleic Acids Res* **38**, 4108-19.
16. Olombrada, M., Rodríguez-Mateos, M., Prieto, D., Pla, J., Remacha, M., Martínez-del-Pozo, A., Gavilanes, J. G., Ballesta, J. P. & García-Ortega, L. (2014). The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by alpha-sarcin of the eukaryotic ribosome. *Biochemistry* **53**, 1545-7.
17. Castaño-Rodríguez, C., Olombrada, M., Partida-Hanon, A., Lacadena, J., Oñaderra, M., Gavilanes, J. G., García-Ortega, L. & Martínez-Del-Pozo, A. (2015). Involvement of loops 2 and 3 of alpha-sarcin on its ribotoxic activity. *Toxicon* **96**, 1-9.
18. Olombrada, M., Herrero-Galán, E., Tello, D., Oñaderra, M., Gavilanes, J. G., Martínez-del-Pozo, A. & García-Ortega, L. (2013). Fungal extracellular ribotoxins as insecticidal agents. *Insect Biochem Mol Biol* **43**, 39-46.

Resultados B: LAS RIBOTOXINAS FÚNGICAS COMO INSECTICIDAS

B1. Las ribotoxinas extracelulares fúngicas como agentes insecticidas

Las ribotoxinas fúngicas se descubrieron hace alrededor de 50 años como agentes antitumorales, si bien, la función que ejercen en la Naturaleza sigue siendo hoy en día una incógnita. El descubrimiento hace unos años de una nueva ribotoxina, llamada hirsutelina A (HtA), que produce el hongo entomopatógeno *Hirsutella thompsonii*, hizo revivir la hipótesis de que las ribotoxinas tuvieran una función insecticida en el contexto biológico del hongo. Lamentablemente, esta ribotoxina es bastante diferente a otros miembros de su familia en su estructura y tamaño, planteando la cuestión de si su actividad insecticida es un caso aislado o si, por el contrario, es una actividad conservada también en otras ribotoxinas. Para responder a esta pregunta, en este trabajo se ha evaluado la actividad insecticida de las ribotoxinas HtA y α-sarcina. Utilizando ribosomas, células en cultivo y larvas de insecto, los resultados obtenidos apuntan a que la actividad insecticida es característica de todas las ribotoxinas, lo que apoyaría la idea de que ésa fuera su principal función.

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Fungal extracellular ribotoxins as insecticidal agents

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ABSTRACT

Fungal ribotoxins were discovered almost 50 years ago as extracellular ribonucleases (RNases) with antitumoral properties. However, the biological function of these toxic proteins has remained elusive. The discovery of the ribotoxin HtA, produced by the invertebrates pathogen *Hirsutella thompsonii*, revived the old proposal that insecticidal activity would be their long searched function. Unfortunately, HtA is rather singular among all ribotoxins known in terms of sequence and structure similarities. Thus, it was intriguing to answer the question of whether HtA is just an exception or, on the contrary, the paradigmatic example of the ribotoxins function. The work presented uses HtA and α-sarcin, the most representative member of the ribotoxins family, to show their strong toxic action against insect larvae and cells.

1. Introduction

Microorganisms populate almost any ecological niche and establish complex and sometimes essential relationships with higher organisms. Consequently, a high diversity of interactions can be found, most of them based on biological mutualisms or antagonisms (Berenbaum and Eisner, 2008). In this regard, fungi constitute a rich source of nitrogen and phosphorous for arthropods and therefore share a long evolutionary history with them. Thus, not only fungi are under constant attack by fungivorous animals such as collembolan, mites and insects (Boddy and Jones, 2008; Ruess and Lussenhop, 2005) but also display frequent mutualistic relationships (Scott et al., 2008), as can be exemplified by fungus farming ants (Currie et al., 1999). Underlying these relationships there is a complex interaction network involving preying, defense, and feeding. Elucidation of these interactions can drive to the discovery and understanding of natural products of unforeseen function (Berenbaum and Eisner, 2008).

Within this idea, it is noticeable that the fungal genus *Hirsutella* contains over 50 fungal species which are known to be entomopathogens. Under in vivo conditions, conidia contact the host, attach to the cuticle, germinate, and penetrate through it (Liu et al., 1995). Along the 1990s, crude filtrates of *Hirsutella thompsonii*, a particular species of this genus, were found to be toxic to a wide variety of arthropods including moth, fly, and mosquito larvae, aphids and mites (Liu et al., 1995, 1996; Omoto and McCoy, 1998; Vey et al., 1993). A toxic protein, Hirsutellin A (HtA), was then isolated from these cultures and proved to show broad pathogenic activity against insects (Krasnoff and Gupta, 1994; Mazet and Vey, 1995). HtA was lethal to *Galleria mellonella* larvae upon injection (Mazet and Vey, 1995) and caused detectable cytopathic effects on *Spodoptera frugiperda* cells (Sf9), inhibiting cell growth (Liu et al., 1995), for example. The ribosomal RNA (rRNA) extracted from these cells contained a small fragment of about 500–600 nt (Liu et al., 1996) resembling the α-fragment produced by fungal ribotoxins upon inactivation of eukaryotic ribosomes (Chan et al., 1983; Endo and Wool, 1982; Endo et al., 1983; Schindler and Davies, 1977). Accordingly, HtA was demonstrated to be a ribotoxin (Herrero-Galán et al., 2008) and subjected to detailed structural and functional characterization (Herrero-Galán et al., 2012a, 2012b; Viegas et al., 2009).

Fungal ribotoxins are extracellular and highly specific ribonucleases which behave as potent inhibitors of protein biosynthesis by being able to inactivate ribosomes from almost any organism (Gasset et al., 1994; Kao et al., 2001; Lacadena et al., 2007; Martínez-Ruiz et al., 2001). They cleave the larger rRNA component at a single phosphodiester bond located within the universally conserved sarcin/ricin loop (SRL) (Chan et al., 1983; Endo and Wool, 1982; Endo et al., 1983; Schindler and Davies, 1977), leading to

Abbreviations: HtA, hirsutellin A; RNases, ribonucleases; SRL, sarcin/ricin loop; TLC, thin layer chromatography.

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complete inactivation of the ribosome and cell death by apoptosis (Olmo et al., 2001). Unfortunately, apart from generalized assertions such as being involved in defense or predation, the biological function of these unique ribonucleases still remains undetermined.

α-Sarcin, restrictocin, and Aspf1 are the most exhaustively characterized ribotoxins (Arruda et al., 1992; García-Ortega et al., 2005; Gasset et al., 1994; Kao et al., 2001; Martínez-Ruiz et al., 2001; Wool, 1997), but many others have been identified and partially characterized within different fungal species (Huang et al., 1997; Lin et al., 1995; Martínez-Ruiz et al., 1999a, 1999b; Parente et al., 1996; Varga and Samson, 2008; Wirth et al., 1997). These studies have shown a high degree of conservation among ribotoxins, as most of those so far characterized display amino acid sequence similarities above 85% (Lacadena et al., 2007). Surprisingly, HtA is a much smaller protein (130 amino acids against 149/150 of the other known ribotoxins) which displays only 25% sequence identity with previously known family members but still contains the same elements of periodic secondary structure and identical arrangement of the active site residues (Boucias et al., 1998; Herrero-Galán et al., 2008; Martínez-Ruiz et al., 1999a). Interestingly, other closely related fungal extracellular RNases, such as RNases T1 and U2, which are only about 100 amino acids long, still show high sequence identity with ribotoxins and contain identical elements of ordered secondary structure. However, they are non-toxic and show low substrate specificity upon cleaving RNA (Martínez-Ruiz et al., 1999a, 2001). Could we then extrapolate HtA insecticidal properties to the whole ribotoxins family? In that case, it would contribute to answer the long time question about the unknown function of this family of toxins (Brandhorst et al., 1996, 2001; Herrero-Galán et al., 2008; Viegas et al., 2009).

In fact, the assignment of an insecticidal function to fungal ribotoxins has been suggested before (Brandhorst et al., 1996, 2001). These authors studied the behavior of the beetle *Carpophilus freemani* against two different *Aspergilli*: The ribotoxin producer *Aspergillus restrictus*, and *Aspergillus nidulans*, which genome does not contain a gene for this type of proteins (Brandhorst et al., 1996). These results showed a significant decrease in feeding on the first of these two fungi when conidia were starting to develop, while no change in the insect behavior was observed against maturing *A. nidulans*. The period of not consumption was coincident with a dramatic build up of restrictocin upon the surfaces of conidia and phialides (Brandhorst and Kenealy, 1992) suggesting that it could deter insects from feeding until conidia were fully formed. Once the spores were mature, the ribotoxin levels would decrease allowing insects to carry spores to new locations for spreading the fungus.

We now show compelling additional evidence of how two of the most different ribotoxins known, α-sarcin and HtA, display similar strong insecticidal properties which are highly dependent on their specific ribonucleolytic activity. Altogether these results are discussed in terms of this insecticidal activity being the biological function of fungal ribotoxins.

2. Materials and methods

2.1. Protein production and purification

Fungal natural wild-type α-sarcin and HtA were produced and isolated as reported before (Herrero-Galán et al., 2008; Martínez-Ruiz et al., 2001). *Escherichia coli* BL21 (DE3) cells, previously cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding plasmid (pINPGαSH137Q), were used to produce the catalytically inactive α-sarcin H137Q mutant, also as previously described (García-Ortega et al., 2000; Lacadena et al., 1994, 1995, 1999). This mutant retains the structural features of the wild-type protein, as well as its ability to interact with membranes, but lacks the characteristic ribonucleolytic activity of ribotoxins (Lacadena et al., 1995, 1999). SDS-PAGE of proteins, Western blots, protein hydrolysis, amino acid analysis, and spectroscopic characterization were performed according to standardized procedures described before (García-Ortega et al., 2000, 2002; Lacadena et al., 1994; Martínez-Ruiz et al., 2001). According to all these criteria, the three proteins used in this study were purified to homogeneity and retained their structural and functional properties.

2.2. Galleria mellonella killing assay

G. mellonella caterpillars in the final instar larval stage were obtained from the company Animal Center (Pobla de Vallbona, Valencia, Spain), stored at 4 °C in the dark and used within 7 days from the day of shipment. Caterpillars with body weights ranging between 0.2 and 0.4 g and absent of any gray markings on the cuticle were employed in all assays. Fifteen randomly chosen caterpillars were used per group in every assay. Results shown are the average of at least three independent assays.

Assays were performed as described (Fuchs et al., 2010; Mylonakis et al., 2005; Mylonakis, 2008). A 10-μL Hamilton syringe was used to inject 8-μL aliquots of the inoculum into the hemocoel of each caterpillar via the last left proleg. The inoculum contained different protein concentrations, ranging between 0 and 50 μM, dissolved in autoclaved 0.9% (w/v) NaCl. After injection, caterpillars were incubated in plastic Petri dishes at 30 °C in the dark. The number of dead caterpillars or pupae formed was scored daily. Caterpillars were considered dead when they displayed no movement in response to touch. Dead larvae were removed from the Petri dish housing the remaining viable larvae. Controls included a group that did not receive any injection in order to monitor the overall quality of the larvae during the course of the experiment and a 0.9% (w/v) NaCl injection group to ensure that death was not due to trauma.

2.3. Insect cells culture and toxicity assays

The insect cell lines *S. frugiperda* (Sf9) and *Trichoplusia ni* (Tni High Five) were cultured as described (Tello et al., 2010) in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker) at 27 °C as indicated by the manufacturer. Toxicity assays were made seeding 80% confluent cells, supplemented with 10 mg/L gentamycin, into 24-well plastic plates and then adding 500 μL of the same medium containing different protein concentrations. Protein solutions were prepared in Insect X-press medium and sterilized by ultrafiltration. Plates were then incubated for 60 h at 27 °C. Dead cells were counted by the dye exclusion method using a Neubauer chamber and a Nikon Eclipse TE 2000-U microscope, after convenient Trypan blue staining of the corresponding cells suspensions.

2.4. Protein biosynthesis inhibition assay using S. frugiperda cells

Sf9 cells were seeded into 24-well plates at a cell density of 10^5 cells/well and were maintained under standard culture conditions up to 80% confluency (2 days). Then, monolayer cultures were incubated in 0.5 mL of fresh medium with serial dilutions of ribotoxin from 10 μM to 1 nM final concentrations. Following 18 h of incubation at 27 °C the medium was replaced by culture medium supplemented with 0.5 μCi/well of [^{3}H]leucine. After 5 h of incubation the medium was removed and cell protein content was precipitated with 5% trichloroacetic acid (TCA) and washed three times with ethanol. The precipitate was dried, dissolved in 200 μL of 0.1 N NaOH, 0.1% SDS and radioactivity was measured in a Beckman LS 3801 liquid scintillation counter. Results are expressed as percentage of incorporated radioactivity relative to samples without protein added.

2.5. Isolation of ribosomes from S. frugiperda cells

Ribosome preparation was obtained from 100% confluency monolayer of Sf9 cells grown in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker). Cells from eight F300 flasks were recovered in 20 mL of cold buffer A (50 mM Tris–HCl pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 5 mM β-mercaptoethanol) supplemented with 0.2 mM PMSF and a proteases inhibitor cocktail. Cell lysis was performed with a French press at 18.000 psi. Ribosome purification followed a standard protocol described before (Powers and Noller, 1991), including a continuous sucrose gradient fractionation. Ribosomes were stored in buffer A, lacking EDTA, at −80 °C.

2.6. Ribonucleolytic activity assays

The specific and unique ribonucleolytic action of ribotoxins on ribosomes results in the cleavage of a single phosphodiester bond located at the SRL with the subsequent release of a characteristic rRNA fragment (the α-fragment) which can be visualized by different means. Thus, one first set of experiments was aimed at detecting the presence of this α-fragment in the rRNA extracted from insect cells previously treated with different amounts of α–sarcin or HtA. With this purpose, cells treated as described in the previous protein biosynthesis inhibition section were recovered in 200 μL of 100 mM HEPES-HCl pH 7.5, pelleted and resuspended again in 200 μL QIAzol lysis reagent (Qiagen). RNA was then extracted from the aqueous phase, following addition of 40 μL of chloroform, and precipitated with isopropanol at RT. Following a 70% ethanol washing step the RNA was finally resuspended in 10 μL of H_2O. This RNA preparation was analyzed using two different approaches (García-Ortega et al., 2010). First, a denaturing 2% (w/v) agarose electrophoresis in MOPS buffer followed by ethidium bromide staining, was used to analyze the rRNA integrity and detect the presence of the α-fragment. Second, the extent and position of cleavage was also determined by poison primer extension as described before. In this case, reverse transcription was performed using the primer 5′-ACCAAATGTCTGAACCTGCGG-3′, which complements the sequence downstream of the SRL in 28S rRNA of *S. frugiperda*. This reaction gave different products for an intact and a cleaved template. The uncleaved rRNA was transcribed up to the first uridine in the sequence due to the ddATP in the extension mixture. The extension of the cleaved template stopped at the cleavage site. The products of reverse transcription were then separated in a 10% denaturing polyacrylamide gel and quantitated with a PhosphorImager (Molecular Dynamics). Percentage of sarcin/ricin cleavage was obtained as $\alpha/(\alpha+28S) \times 100$, where α and 28S correspond to the amounts of α-fragment and intact RNA found in each case, respectively.

In a second set of experiments, identical analyses were made but now ribotoxins were assayed against isolated Sf9 ribosomes as substrates. Within this purpose, reactions were performed in 25 μL of 30 mM Tris–HCl pH 7.5, 150 mM NH_4Cl, 5 mM $MgCl_2$, 2 mM EDTA and 4 mM β-mercaptoethanol. Ribosome concentration was 0.2 μM. Incubation times varied from 30 s to 90 min and ribotoxin concentrations from 10 to 200 nM. In order to stop the reaction, 125 μL of 0.36 M NaAc, 1.1% (w/v) SDS were added and then RNA was extracted with phenol/chloroform and precipitated with ethanol. The RNA obtained was analyzed by denaturing agarose electrophoresis and poison primer extension as described above.

2.7. Leakage of aqueous content from lipid vesicles

The breakdown of the permeability barrier of lipid bilayers can be analyzed using an assay employing the fluorescence probe 8-aminonaphtalene1,3,6-trisulfonic acid (ANTS) and its colisional quencher N,N-p-xilene-bispyridinium bromide (DPX) (Ellens et al., 1985, 1986). When they are encapsulated into lipid vesicles, the release of the intravesicular content to the external medium results in dilution of probe and quencher, with the concomitant increase in ANTS fluorescence. Plasma membrane of Sf9 cells was isolated and separated from the endoplasmic reticulum and Golgi apparatus fractions using a discontinuous sucrose gradient (Hu and Kaplan, 2000). Total lipids from the plasma membrane were then isolated using the following procedure (Folch et al., 1957): The plasma membrane fraction was centrifuged for 1 h at 20,000 rpm in a TLA120.1 rotor, resuspended in 3 mL of chloroform/methanol (2:1 v:v) and shaken gently at room temperature for 1 h. After washing with 0.2 volume of 0.9% NaCl solution, the mixture was centrifuged at low speed to separate the two phases. The lower chloroform phase was recovered, evaporated under a stream of nitrogen and stored at −20 °C. Analysis of the phospholipids content in this cell membrane preparation was performed using thin-layer chromatography (TLC) plates (silica gel 60, 20 × 20 cm, Merck) as described (Gavilanes et al., 1981). TLC fractionation of the lipid plasma membrane extracts revealed high amounts of phosphatidylcholine and phosphatidylethanolamine (data not shown) in good accordance with the high abundance of these phospholipids in total lipidic extracts of Sf9 cells (Yeh et al., 1997). This pool of insect lipids was used to prepare vesicles as described before (De los Ríos et al., 1998; Herrero-Galán et al., 2008; Martínez-Ruiz et al., 2001) by hydrating dry lipid films with 15 mM Tris–HCl, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, for 60 min at 37 °C and in the presence of 12.5 mM ANTS and 45 mM DPX. The lipid suspension was then subjected to five cycles of extrusion through two stacked 0.1 μm (pore diameter) polycarbonate membranes (Mancheño et al., 1994; Martínez-Ruiz et al., 2001). Unencapsulated material was separated from the vesicles by gel filtration on a Sephadex G-75 column in the same buffer. Phospholipid concentration was determined as described (Barlett, 1959). Leakage of vesicle aqueous content was then measured by adding small volumes of freshly prepared solutions of proteins in the corresponding buffer and recording the emission above 530 nm (using a 3–68 Corning cutoff filter) upon excitation at 386 nm. Polarizers were used to eliminate potential contribution of sample turbidity to the signal registered. The extent of leakage was defined as *Relative leakage* $= (F_P - F_0)/(F_T - F_0)$, where F_P and F_0 are the fluorescence intensity values after and before the addition of protein, respectively, and F_T is the value after addition of 10% Triton X-100 (total vesicle lysis) (Martínez-Ruiz et al., 2001).

2.8. Statistical analysis

Statistical analyses were carried out by using the statistics utility of Sigma Plot v11.2 Program (Systat Software, Erkrath, Germany). The normality of the data was checked by using Shapiro–Wilk test and equal variance test. Percentage values were Arcsine-Square Root transformed before statistical analysis. ANOVA analyses were used for comparison of multiple groups of factors. Statistical significance was considered to be achieved at the $p < 0.05$ level.

3. Results

3.1. Galleria mellonella killing assay

Injection of either fungal natural wild-type α-sarcin or HtA resulted in larvae death. Toxicity was dependent on ribotoxin concentration, HtA being more effective in terms of less amount of protein needed to produce the same mortality levels (Fig. 1). Upon

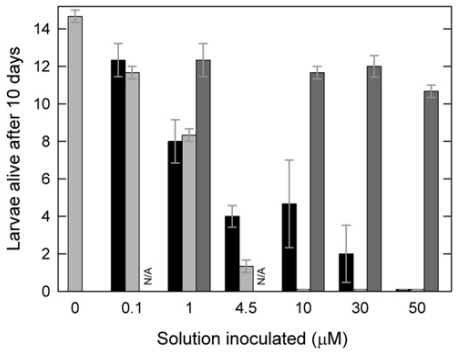

Fig. 1. - G. mellonella larvae survival after 10 days of inoculation with 8.0 μL of either natural fungal wild-type α-sarcin (black bars), HtA (gray bars) or the catalytically inactive α-sarcin H137Q mutant (dark gray bars). The 0 bar corresponds to control larvae inoculated only with 0.9% NaCl. Fifteen larvae were injected for each protein concentration employed. Results are the average ± SD of three different sets of experiments. ANOVA analysis revealed that there was a statistically significant difference between the treatment with α-sarcin or HtA at the concentrations assayed respect to the control (NaCl treatment, $p < 0.001$), but not in the case of the α sarcin H137Q mutant ($p = 0.598$).

toxin injection larvae begun to lose mobility. A brownish coloration slowly appeared, becoming dark brown or even black, upon death. These color changes were most probably due to an overactivation of the phenoloxidase cascade, as it has been described as one of the key defense mechanisms against pathogens in insects (Soderhall and Cerenius, 1998). Differences in larvae survival were especially evident after ten days of incubation at 30 °C (Fig. 1). On the other hand, injection of the catalytically inactive α-sarcin H137Q mutant had almost a negligible effect on survival for identical doses and incubation times (Fig. 1), suggesting that the highly specific RNase activity of ribotoxins is important for their insecticidal lethal action. The toxic effect of ribotoxins against *G. mellonella* larvae was also evident in terms of pupation delay (data not shown).

3.2. Toxic effect on insect cell lines in culture

Both ribotoxins, α-sarcin and HtA, displayed toxicity when assayed against two different insect cell lines (Fig. 2), being slightly more effective against *S. frugiperda* cells (Sf9) than against *Trichoplusia ni* (Tni High Five) cells, in terms of the estimated IC_{50} values.

Given the higher sensibility of *S. frugiperda* cells to the action of both ribotoxins, this cell line was chosen to further study the effect of the two proteins at the molecular level. Both α-sarcin and HtA showed a dramatic effect on the inhibition of in vivo protein biosynthesis with IC_{50} values in the nanomolar range (Fig. 3). These values were in the same order of magnitude as those obtained from cell viability assays, considering the different nature of both experiments. On the other hand, they were around two orders of magnitude smaller than those obtained for human rhabdomyosarcoma cells commonly used in the standard assay for evaluating ribotoxins antitumoral activity (Herrero-Galán et al., 2008; Olmo et al., 2001; Turnay et al., 1993). The production of α-fragment in these cultures was analyzed by poison primer extension once total RNA was isolated (Fig. 4). The result showed that 28S rRNA was cleaved by both ribotoxins with an identical pattern to that described for *E. coli* ribosomes (García-Ortega et al., 2010). Thus, the extent of cleavage of the sarcin/ricin loop was consistent with the inhibition of protein synthesis, supporting the notion that the specific ribonucleolytic action of α-sarcin and HtA is the cause for their toxicity against this insect cell line.

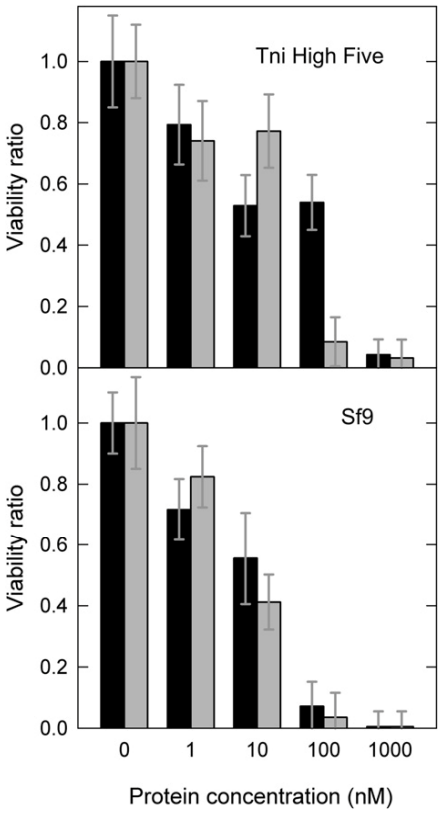

Fig. 2. - Toxic effect of the addition of increasing ribotoxin concentrations to the culture medium of two different insect cell lines. Results are expressed as viability ratios (proportion of cells remaining alive for an initial population of 3×10^5 cells) ± SD after 60 h of incubation in the presence of α-sarcin (black bars) or HtA (gray bars). ANOVA analysis of Arcsine-Square Root transformed values was performed. No significant differences were observed for the toxicity of both ribotoxins on the two insect cell lines, except for Tni High Five cells at 10 and 100 nM toxin concentration ($p = 0.002$ and $p < 0.001$, respectively), and Sf9 cells at 10 nM ($p = 0.025$).

3.3. Ribotoxin effect against purified insect ribosomes

Insect ribosomes were isolated from Sf9 cells by a two step purification protocol based on a salt wash and a continuous sucrose

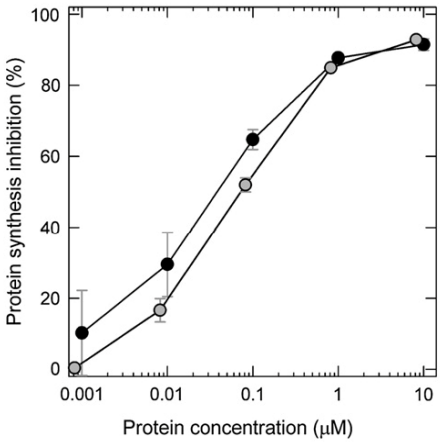

Fig. 3. - Protein biosynthesis inhibition ± SD in Sf9 insect cells cultured in the presence of different α-sarcin (black dots) or HtA (gray dots) concentrations. Results are the average of three different sets of experiments. ANOVA analysis of Arcsine-Square Root transformed values revealed that the effect of both proteins was significantly different ($p = 0.003$) in the overall concentration range.

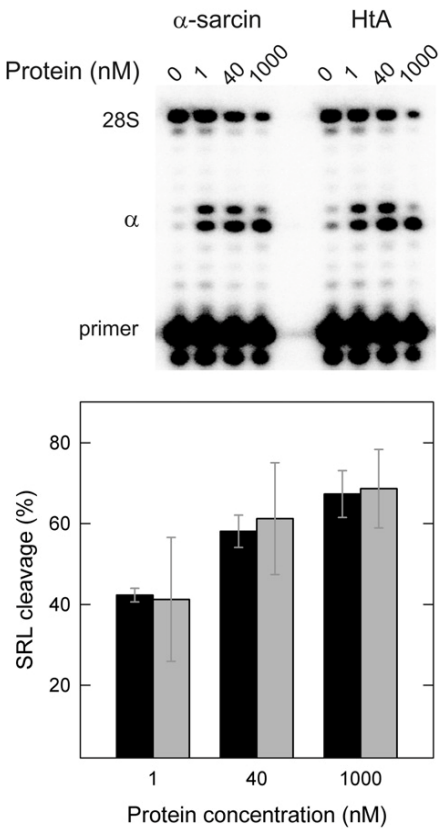

Fig. 4. - rRNA analysis of Sf9 insect cells treated with different concentrations of ribotoxin. The poison primer extension assay showed two additional bands corresponding to the α-fragment (α) when cells were incubated with the ribotoxin. Quantification was expressed as the percentage of specific cleavage at the sarcin/ricin loop obtained. Black bars and gray bars correspond to α-sarcin and HtA, respectively. Results are the average ± SD of three different sets of experiments. ANOVA analysis of Arcsine-Square Root transformed values showed no significant differences between both toxins.

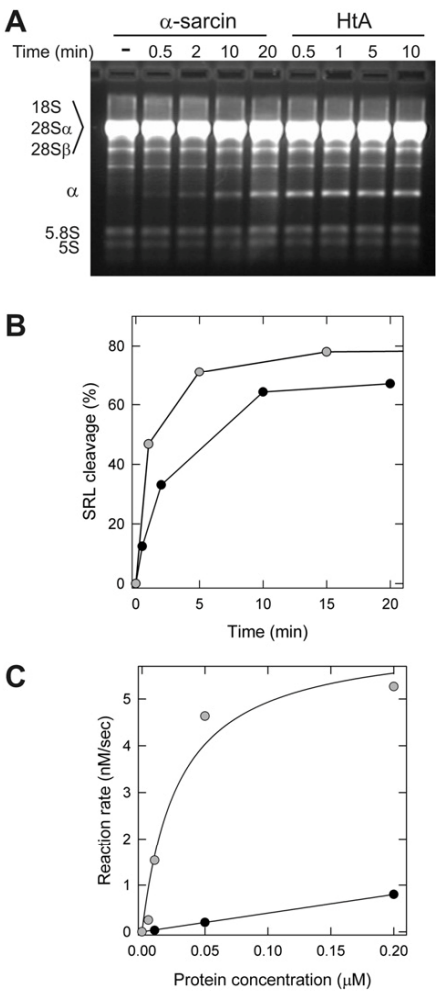

Fig. 5. - α-Sarcin (black dots) or HtA (gray dots) activity on isolated Sf9 ribosomes. (**A**) Integrity of ribosomal RNA after ribotoxin treatment analyzed by denaturing agarose gel electrophoresis. Positions corresponding to the different rRNA molecules, α-fragment included, are indicated. (**B**) Two time courses of SRL cleavage quantified by primer extension analysis are shown: α-Sarcin 0.2 μM and HtA 10 nM. (**C**) Dependence of the reaction rate obtained from different time courses as shown in B with ribotoxin concentration.

gradient, as described elsewhere for prokaryotic and eukaryotic organisms (Spedding, 1990). Ribosomal RNA analysis showed the unique pattern for these ribosomes of an apparent single band in agarose gels (Fig. 5A). This is a consequence of a natural hidden break in the 28S rRNA without affecting the ribosome integrity (Winnebeck et al., 2010).

α-Sarcin and HtA treatment of these ribosomes produced an additional RNA band, the known α-fragment (Fig. 5A), as a result of their specific activity against these substrates. Different protein concentrations and reaction times were analyzed (Fig. 5B). Quantification of the extent of specific cleavage by primer extension showed an important difference between the activities of both ribotoxins. HtA was dramatically more efficient than α-sarcin (Fig. 5C), with an apparent affinity constant in the nanomolar range in these conditions.

3.4. Leakage of aqueous content from lipid vesicles

Regardless of their ribonucleolytic activity, HtA and α-sarcin also show significant differences when interacting with biological membrane model systems (Herrero-Galán et al., 2008). HtA membrane permeabilizing activity is higher than that of α-sarcin. In this case, we studied the ability of both proteins to promote leakage of aqueous content from vesicles made of lipids extracted from the plasmatic membrane of Sf9 cells. The results obtained confirmed that both toxins retained the membrane interaction ability in this insect model system but also that HtA was more effective as a leakage inducing agent (Fig. 6).

4. Discussion

The first fungal ribotoxin was discovered almost 50 years ago during a screening program of the Michigan Department of Health searching for antibiotics and antitumor agents. Culture filtrates of a mold isolated from a sample of farm soil were found to contain a substance inhibitory to both sarcoma and carcinoma induced in mice (Olson et al., 1965). The mold was identified as *Aspergillus giganteus* MDH18894, and the molecule responsible for these effects proved to be a protein, named α-sarcin after its anti-sarcoma activity (Olson and Goerner, 1965). Not much later two more ribotoxins were discovered but further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses (Roga et al., 1971) and caused the abandonment of their study. A few years later it was demonstrated that they

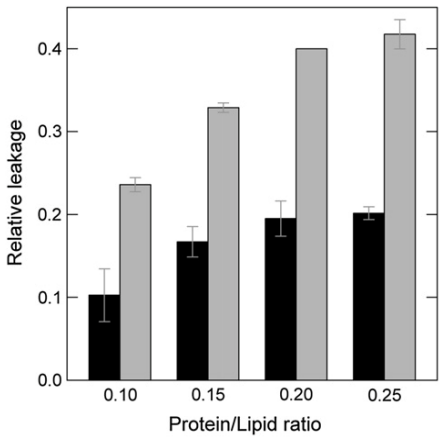

Fig. 6. - Leakage of aqueous content of vesicles prepared with lipids extracted from cultured Sf9 cells upon addition of different α-sarcin (black bars) or HtA (gray bars) concentrations. Relative leakage, considering that produced by detergent as unit, is expressed as a function of protein/lipid molar ratios. Results are the average of two experiments. ANOVA analysis of Arcsine-Square Root transformed leakage values showed significant differences between both toxins ($p < 0.001$).

inhibited protein biosynthesis by specifically cleaving a unique phosphodiester bond of the large rRNA fragment (Endo and Wool, 1982; Schindler and Davies, 1977) located at an evolutionarily conserved site with important roles in ribosome function. This observation prompted the development of their molecular characterization (Lacadena et al., 2007). Much later, more systematic analysis of fungal genomes revealed that ribotoxins were a more widespread group of proteins within the filamentous fungi than had been previously believed (Lin et al., 1995; Martínez-Ruiz et al., 1999b; Varga and Samson, 2008). All this exhaustive work dealing with the characterization of their mechanism of action at the molecular level has not only helped to understand the basis of their antitumoral action (Lacadena et al., 2007) but also has established the principles to transform some of them into engineered chimeras with potential therapeutic uses (Carreras-Sangrà et al., 2008, 2012).

It seems clear, however, that fungi are not secreting these lethal toxins with the purpose of combating mammalian cancer. In fact, the real biological function of these toxic proteins has remained elusive. The discovery of the insecticidal protein HtA and the demonstration that it was another member of the ribotoxins family (Herrero-Galán et al., 2008) opened the door to speculate about insecticidal activity being their so long searched function. A function that had been already suggested by some other authors (Brandhorst et al., 1996, 2001) but that was far from being proven in full. Furthermore, HtA is the most singular of all ribotoxins known, given its small size and its much lower degree of sequence identity with the other members of the family. Thus, it was intriguing to answer the question of whether HtA was just an exception or, on the contrary, a paradigmatic example of their function. The present study was therefore performed with the aim of discerning this dilemma, comparing HtA and α-sarcin, the most representative member of the ribotoxins family.

The results obtained show how both ribotoxins are highly toxic against *G. mellonella* larvae (Fig. 1) and insect cells in culture (Figs. 2 and 3). Indeed, their insect killing activity is linked to their specific ribonucleolytic action on insect ribosomes as proven by the production of the α-fragment within treated cells (Fig. 4). Within this same idea, the ribonucleolytically inactive H137Q α-sarcin variant is also much less toxic when assayed against larvae (Fig. 1). This toxicity is two orders of magnitude higher than that one described for their antitumoral activity, supporting the biological function of this family of proteins as insecticidal agents. The explanation lies precisely at the heart of the unique action of ribotoxins. Thus, in addition to their ribonucleolytic activity, ribotoxins are extracellular RNases that cross lipid membranes to reach their target in the absence of any known protein receptor (Oñaderra et al., 1993; Gasset et al., 1994; Martínez-Ruiz et al., 2001). Consequently, although any ribosome could be potentially inactivated by these proteins, due to the universal conservativeness of the SRL, ribotoxins have been described as especially active on transformed or virus-infected cells (Fernández-Puentes and Carrasco, 1980; Olmo et al., 2001; Olson et al., 1965). This observation has been explained in terms of an altered permeability of these cells combined with the ability of ribotoxins to interact with acid phospholipid-containing membranes (Gasset et al., 1989, 1990, 1994; Herrero-Galán et al., 2008, 2012b; Martínez-Ruiz et al., 2001; Olmo et al., 2001). Insect cells have a different plasma membrane composition from mammalian cells due to their higher content of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol and a significant lower cholesterol/phospholipid ratio (Marheineke et al., 1998). Therefore insect plasma membranes are thinner and probably more fluid than those in mammalian cells being better candidates as targets for ribotoxins.

Unexpectedly both proteins showed large differences when assayed against isolated insect ribosomes (Fig. 5). In the conditions assayed, HtA is significantly more active than α-sarcin, although both proteins still recognize specifically the SRL as proven by the release of the α-fragment (Fig. 5A). The apparent discrepancy of both proteins displaying very similar toxicity against larvae and cells but different affinities against ribosomes is consistent with passage across the cell membrane being the rate limiting step (Olmo et al., 2001; Turnay et al., 1993). Thus, considering that cellular internalization takes hours to complete, the kinetic and affinity differences observed against purified ribosomes (Fig. 5) would be negligible in establishing a different cytotoxic activity when employed against intact cells. Although HtA still shows higher membrane permeabilizing activity than α-sarcin when tested against model vesicles made of insect plasmatic lipid membranes (Fig. 6), this difference is not large enough to expect a significant variation in the cytotoxic activity of both ribotoxins.

The different behavior of HtA and α-sarcin when interacting with ribosomes is however very interesting at the molecular level. This action has been now quantified not only with insect ribosomes but also with ribosomes from other origins like yeast or bacteria (unpublished results). It has been so far presumed that the extra loops in the structure of ribotoxins were responsible for their specific ribosomal recognition when compared with their structurally related non-toxic T1-like ribonucleases. Considering that HtA essentially differs from the rest of the family in having different and shorter non ordered loops, the detailed analysis of the mechanisms of ribosome interaction of both HtA and α-sarcin is now a challenging question. This means that not only their specific ribonucleolytic activity and their lipid interaction are important factors for their specificity of action but also aspects like the efficiency of their natural biosynthesis and extracellular export, their stability in a particular environment or the accessibility to their target. Supporting these ideas is the fact that *Aspergillus*, the main producer of ribotoxins, and *Hirsutella* are very different fungal geni which also thrive in very different environments.

In summary, the so long studied antitumoral action of fungal ribotoxins seems to be just a side effect of their insecticidal function. Depending on the environment and the producing fungus this toxicity could be involved in protecting from predation of different arthropods or in parasitism. So far we have proved their biological toxicity against insects. Further studies in closer to nature niches will precise their natural function. Within this idea, the study of

ribotoxins would constitute a nice example of how understanding the molecular mechanism of toxic proteins can help to yield beneficial therapeutic uses of the proteins involved in that characterization.

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References

Arruda, L.K., Mann, B.J., Chapman, M.D., 1992. Selective expression of a major allergen and cytotoxin, Aspf1, in *Aspergillus fumigatus*. Implications for the immunopathogenesis of *Aspergillus*-related diseases. J. Immunol. 149, 3354–3359.

Barlett, G.R., 1959. Colorimetric assay methods for free and phosphorylated glyceric acids. J. Biol. Chem. 234, 466–468.

Berenbaum, M.R., Eisner, T., 2008. Bugs' bugs. Science 322, 52–53.

Boddy, L., Jones, T.H., 2008. Interactions between basidiomycota and invertebrates. In: Boddy, L., et al. (Eds.), Ecology of Saprotrophic Basidiomycetes. Academic Press, pp. 155–179.

Boucias, D.G., Farmerie, W.G., Pendland, J.C., 1998. Cloning and sequencing of cDNA of the insecticidal toxin hirsutellin A. J. Invertebr. Pathol. 72 (3), 258–261.

Brandhorst, T.T., Kenealy, W.R., 1992. Production and localization of restrictocin in *Aspergillus restrictus*. J. Gen. Microbiol. 138, 1429–1435.

Brandhorst, T., Dowd, P.F., Kenealy, W.R., 1996. The ribosome-inactivating protein restrictocin deters insect feeding on *Aspergillus restrictus*. Microbiology 142, 1551–1556.

Brandhorst, T., Dowd, P.F., Kenealy, W.R., 2001. The effect of fungal ribosome inactivating proteins upon feeding choice in *C. freemani*, and indications of a mutualistic relationship with *A. restrictus*. Mycopathologia 152, 155–158.

Carreras-Sangrà, N., Álvarez-García, E., Herrero-Galán, E., Tomé, J., Lacadena, J., Alegre-Cebollada, J., Oñaderra, M., Gavilanes, J.G., Martínez-del-Pozo, A., 2008. The therapeutic potential of fungal ribotoxins. Curr. Pharm. Biotechnol. 9, 153–160.

Carreras-Sangrà, N., Tomé-Amat, J., García-Ortega, L., Batt, C.A., Oñaderra, M., Martínez-del-Pozo, A., Gavilanes, J.G., Lacadena, J., 2012. Production and characterization of a colon cancer specific immunotoxin based on the fungal ribotoxin α-sarcin. Protein Engineer. Des. Sel. 25 (8), 425–435.

Chan, Y.L., Endo, Y., Wool, I.G., 1983. The sequence of the nucleotides at the α-sarcin cleavage site in rat 28S ribosomal ribonucleic acid. J. Biol. Chem. 258, 12768–12770.

Currie, C.R., Mueller, U.G., Malloch, D., 1999. The agricultural pathology of ant fungus gardens. Proc. Natl. Acad. Sci. U. S. A. 96, 7998–8002.

De los Ríos, V., Mancheño, J.M., Lanio, M.E., Oñaderra, M., Gavilanes, J.G., 1998. Mechanism of the leakage induced on lipid model membranes by the hemolytic protein sticholysin II from the sea anemone *Stichodactyla helianthus*. Eur. J. Biochem. 252, 284–289.

Ellens, H., Bentz, J., Szoka, F.C., 1985. Proton- and calcium-induced fusion and destabilization of liposomes. Biochemistry 24, 3099–3106.

Ellens, H., Bentz, J., Szoka, F.C., 1986. Destabilization of phosphatidylethanolamine liposomes at the hexagonal phase transition temperature. Biochemistry 25, 285–294.

Endo, Y., Wool, I.G., 1982. The site of action of α-sarcin on eukaryotic ribosomes. The sequence at the α-sarcin cleavage site in 28S ribosomal ribonucleic acid. J. Biol. Chem. 257, 9054–9060.

Endo, Y., Huber, P.W., Wool, I.G., 1983. The ribonuclease activity of the cytotoxin α-sarcin. The characteristics of the enzymatic activity of α-sarcin with ribosomes and ribonucleic acids as substrates. J. Biol. Chem. 258, 2662–2667.

Fernández-Puentes, C., Carrasco, L., 1980. Viral infection permeabilizes mammalian cells to protein toxins. Cell 20, 769–775.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Fuchs, B.B., O'Brien, E., Khoury, J.B., Mylonakis, E., 2010. Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. Virulence 1, 475–482.

García-Ortega, L., Lacadena, J., Lacadena, V., Masip, M., de Antonio, C., Martínez-Ruiz, A., Martínez-del-Pozo, A., 2000. The solubility of the ribotoxin α-sarcin, produced as a recombinant protein in *Escherichia coli*, is increased in the presence of thioredoxin. Lett. Appl. Microbiol. 30, 298–302.

García-Ortega, L., Masip, M., Mancheño, J.M., Oñaderra, M., Lizarbe, M.A., García-Mayoral, M.F., Bruix, M., Martínez-del-Pozo, A., Gavilanes, J.G., 2002. Deletion of the NH_2- terminal β-hairpin of the ribotoxin α-sarcin produces a non-toxic but active ribonuclease. J. Biol. Chem. 277, 18632–18639.

García-Ortega, L., Lacadena, J., Villalba, M., Rodríguez, R., Crespo, J.F., Rodríguez, J., Pascual, C., Olmo, N., Oñaderra, M., Martínez-del-Pozo, A., Gavilanes, J.G., 2005. Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Aspf1 displaying reduced IgE binding. FEBS J. 272, 2536–2544.

García-Ortega, L., Álvarez-García, E., Gavilanes, J.G., Martínez-del-Pozo, A., Joseph, S., 2010. Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. Nucleic Acids Res. 38, 4108–4119.

Gasset, M., Martínez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1989. Study of the interaction between the antitumour protein α-sarcin and phospholipid vesicles. Biochem. J. 258, 569–575.

Gasset, M., Oñaderra, M., Thomas, P.G., Gavilanes, J.G., 1990. Fusion of phospholipid vesicles produced by the anti-tumour protein α-sarcin. Biochem. J. 265, 815–822.

Gasset, M., Mancheño, J.M., Lacadena, J., Turnay, J., Olmo, N., Lizarbe, M.A., Martínez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1994. α-Sarcin, a ribosome-inactivating protein that translocates across the membrane of phospholipid vesicles. Curr. Top. Pept. Protein Res. 1, 99–104.

Gavilanes, J.G., Lizarbe, M.A., Municio, A.M., Oñaderra, M., 1981. Fluorescence studies on the lipoprotein complex of the fatty acid synthetase from the insect *Ceratitis capitata*. Biochemistry 20, 5689–5694.

Herrero-Galán, E., Lacadena, J., Martínez-del-Pozo, A., Boucias, D.G., Olmo, N., Oñaderra, M., Gavilanes, J.G., 2008. The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. Proteins 72, 217–228.

Herrero-Galán, E., García-Ortega, L., Lacadena, J., Martínez-Del-Pozo, A., Olmo, N., Gavilanes, J.G., Oñaderra, M., 2012a. Implication of an Asp residue in the ribonucleolytic activity of hirsutellin A reveals new electrostatic interactions at the active site of ribotoxins. Biochimie 94, 427–433.

Herrero-Galán, E., García-Ortega, L., Lacadena, J., Martínez-del-Pozo, A., Olmo, N., Gavilanes, J.G., Oñaderra, M., 2012b. A non-cytotoxic but ribonucleolytically specific ribotoxin variant. Implication of tryptophan residues in hirsutellin A. Biol. Chem. 393, 449–456.

Hu, Y., Kaplan, J.H., 2000. Site-directed chemical labeling of extracellular loops in a membrane protein. J. Biol. Chem. 275, 19185–19191.

Huang, K.-C., Hwang, Y.Y., Hwu, L., Lin, A., 1997. Characterization of a new ribotoxin gene (c-sar) from *Aspergillus clavatus*. Toxicon 35, 383–392.

Kao, R., Martínez-Ruiz, A., Martínez-del-Pozo, A., Crameri, R., Davies, J., 2001. Mitogillin and related fungal ribotoxins. Methods Enzymol. 341, 324–335.

Krasnoff, S.B., Gupta, S., 1994. Identification of the antibiotic phomalactone from the entomopathogenic fungus, *Hirsutella thompsonii var. synnematosa*. J. Chem. Ecol. 20, 293–302.

Lacadena, J., Martínez-del-Pozo, A., Barbero, J.L., Mancheño, J.M., Gasset, M., Oñaderra, M., López-Otín, C., Ortega, S., García, J., Gavilanes, J.G., 1994. Overproduction and purification of biologically active native fungal α-sarcin in *Escherichia coli*. Gene 142, 147–151.

Lacadena, J., Mancheño, J.M., Martínez-Ruiz, A., Martínez-del-Pozo, A., Gasset, M., Oñaderra, M., Gavilanes, J.G., 1995. Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin. Biochem. J. 309, 581–586.

Lacadena, J., Martínez-del-Pozo, A., Martínez-Ruiz, A., Pérez-Cañadillas, J.M., Bruix, M., Mancheño, J.M., Oñaderra, M., Gavilanes, J.G., 1999. Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin α-sarcin. Proteins 37, 474–484.

Lacadena, J., Álvarez-García, E., Carreras-Sangrà, N., Herrero-Galán, E., Alegre-Cebollada, J., García-Ortega, L., Oñaderra, M., Gavilanes, J.G., Martínez-del-Pozo, A., 2007. Fungal ribotoxins: molecular dissection of a family of natural killers. FEMS Microbiol. Rev. 31, 212–237.

Lin, A., Huang, K.C., Hwu, L., Tzean, S.S., 1995. Production of type II ribotoxins by Aspergillus species and related fungi in Taiwan. Toxicon 33, 105–110.

Liu, W.Z., Boucias, D.G., McCoy, C.W., 1995. Extraction and characterization of the insecticidal toxin hirsutellin A produced by *Hirsutella thompsonii* var. Thompsonii. Exp. Mycol. 19, 254–262.

Liu, J.C., Boucias, D.G., Pendland, J.C., Liu, W.Z., Maruniak, J., 1996. The mode of action of hirsutellin A on eukaryotic cells. J. Invertebr. Pathol. 67, 224–228.

Mancheño, J.M., Gasset, M., Lacadena, J., Ramón, F., Martínez-del-Pozo, A., Oñaderra, M., Gavilanes, J.G., 1994. Kinetic study of the aggregation and lipid mixing produced by α-sarcin on phosphatidylglycerol and phosphatidylserine vesicles: stopped-flow light scattering and fluorescence energy transfer measurements. Biophys. J. 67, 1117–1125.

Marheineke, K., Grünewald, S., Christie, W., Reiländer, H., 1998. Lipid composition of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) insect cells used for baculovirus infection. FEBS Lett. 441, 49–52.

Martínez-Ruiz, A., Martínez-del-Pozo, A., Lacadena, J., Oñaderra, M., Gavilanes, J.G., 1999a. Hirsutellin A displays significant homology to microbial extracellular ribonucleases. J. Invertebr. Pathol. 74, 96–97.

Martínez-Ruiz, A., Kao, R., Davies, J., Martínez-del-Pozo, A., 1999b. Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. Toxicon 37, 1549–1563.

Martínez-Ruiz, A., García-Ortega, L., Kao, R., Lacadena, J., Oñaderra, M., Mancheño, J.M., Davies, J., Martínez-del-Pozo, A., Gavilanes, J.G., 2001. RNase U2 and α-sarcin: a study of relationships. Methods Enzymol. 341, 335–351.

Mazet, I., Vey, A., 1995. Hirsutellin A, a toxic protein produced *in vitro* by *Hirsutella thompsonii*. Microbiology 141, 1343–1348.

Mylonakis, E., 2008. *Galleria mellonella* and the study of fungal pathogenesis: making the case for another genetically tractable model host. Mycopathologia 165, 1–3.

Mylonakis, E., Moreno, R., El Khoury, J.B., Idnurm, A., Heitman, J., Calderwood, S.B., Ausubel, F.M., Diener, A., 2005. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. Infect. Immun. 73, 3842–3850.

Olmo, N., Turnay, J., González de Buitrago, G., López de Silanes, I., Gavilanes, J.G., Lizarbe, M.A., 2001. Cytotoxic mechanism of the ribotoxin α-sarcin. Induction of cell death via apoptosis. Eur. J. Biochem. 268, 2113–2123.

Olson, B.H., Goerner, G.L., 1965. α-Sarcin, a new antitumour agent. I. Isolation, purification, chemical composition, and the identity of a new amino acid. Appl. Microbiol. 13, 314–321.

Olson, B.H., Jennings, J.C., Roga, V., Junek, A.J., Schuurmans, D.M., 1965. α-Sarcin, a new antitumour agent. II. Fermentation and antitumour spectrum. Appl. Microbiol. 13, 322–326.

Omoto, C., McCoy, C.W., 1998. Toxicity of purified fungal toxin hirsutellin A to the citrus rust mite *Phyllocoptruta oleivora* (Ash.). J. Invertebr. Pathol. *72, 319–322.*

Oñaderra, M., Mancheño, J.M., Gasset, M., Lacadena, J., Schiavo, G., Martínez del Pozo, A., Gavilanes, J.G., 1993. Translocation of α-sarcin across the lipid bilayer of asolectin vesicles. Biochem. J. 295, 221–225.

Parente, D., Raucci, G., Celano, B., Pacilli, A., Zanoni, L., Canevari, S., Adobati, E., Colnaghi, M.I., Dosio, F., Arpicco, S., Cattel, L., Mele, A., De Santis, R., 1996. Clavin, a type-1 ribosome-inactivating protein from Aspergillus clavatus IFO 8605. cDNA isolation, heterologous expression, biochemical and biological characterization of the recombinant protein. Eur. J. Biochem. 239 (2), 272–280.

Powers, T., Noller, H.F., 1991. A functional pseudoknot in 16S ribosomal RNA. EMBO J. 10, 2203–2214.

Roga, V., Hedeman, L.P., Olson, B.H., 1971. Evaluation of mitogillin (NSC-69529) in the treatment of naturally occurring canine neoplasms. Cancer Chemother. Rep. 55, 101–113.

Ruess, L., Lussenhop, J., 2005. Trophic interactions of fungi and animals. In: Dighton, J., et al. (Eds.), The Fungal Community – Its Organization and Role in the Ecosystem. CRC Press, Boca Raton, pp. 581–598.

Schindler, D.G., Davies, J.E., 1977. Specific cleavage of ribosomal RNA caused by α-sarcin. Nucleic Acids Res. 4, 1097–1110.

Scott, J.J., Oh, D.C., Yuceer, M.C., Klepzig, K.D., Clardy, J., Currie, C.R., 2008. Bacterial protection of beetle-fungus mutualism. Science 322, 63.

Soderhall, K., Cerenius, L., 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. 10, 23–28.

Spedding, G., 1990. Isolation and analysis of ribosomes from prokaryotes, eukaryotes, and organelles. In: Spedding, G. (Ed.), Ribosomes and Protein Synthesis: A Practical Approach. Oxford University Press, Oxford, UK, pp. 1–27.

Tello, D., Rodríguez-Rodríguez, M., Yélamos, B., Gómez-Gutiérrez, J., Ortega, S., Pacheco, B., Peterson, D.L., Gavilanes, F., 2010. Expression and structural properties of a chimeric protein based on the ectodomains of E1 and E2 hepatitis C virus envelope glycoproteins. Protein Expr. Purif. 71, 123–131.

Turnay, J., Olmo, N., Jiménez, A., Lizarbe, M.A., Gavilanes, J.G., 1993. Kinetic study of the cytotoxic effect of α–sarcin, a ribosome inactivating protein from Aspergillus giganteus, on tumour cell lines: protein biosynthesis inhibition and cell binding. Mol. Cell. Biochem. 122, 39–47.

Varga, J., Samson, R.A., 2008. Ribotoxin genes in isolates of Aspergillus section Clavati. Antonie Van Leeuwenhoek 94, 481–485.

Vey, A., Quiot, J.M., Mazet, I., McCoy, C.W., 1993. Toxicity and pathology of crude broth filtrate produced by *Hirsutella thompsonii var. thompsonii* in shake culture. J. Invertebr. Pathol. 61, 131–137.

Viegas, A., Herrero-Galán, E., Oñaderra, M., Macedo, A.L., Bruix, M., 2009. Solution structure of hirsutellin A–new insights into the active site and interacting interfaces of ribotoxins. FEBS J. 276, 2381–2390.

Winnebeck, E.C., Millar, C.D., Warman, G.R., 2010. Why does insect RNA look degraded? J. Insect Sci. 10, 159.

Wirth, J., Martínez-del-Pozo, A., Mancheño, J.M., Martínez-Ruiz, A., Lacadena, J., Oñaderra, M., Gavilanes, J.G., 1997. Sequence determination and molecular characterization of gigantin, a cytotoxic protein produced by the mould *Aspergillus giganteus* IFO 5818. Arch. Biochem. Biophys. 343, 188–193.

Wool, I.G., 1997. Structure and mechanism of action of cytotoxic ribonuclease α-sarcin. In: D'Alessio, G., Riordan, J.F. (Eds.), Ribonucleases. Structures and Functions. Academic Press, New York, pp. 131–162.

Yeh, L.H., Bajpai, R.K., Sun, G.Y., 1997. Membrane lipid metabolism and phospholipase activity in insect Spodoptera frugiperda 9 ovarian cells. Lipids 32 (5), 481–487.

B2. Ribotoxinas fúngicas: Armas naturales de origen proteico frente a insectos

Las ribotoxinas son RNasas fúngicas extracelulares que poseen una elevada toxicidad debido a su habilidad para penetrar en las células diana e inactivar el ribosoma de manera muy específica. El papel que juegan estas proteínas en los hongos que las producen no se conoce en detalle. No obstante, los resultados mostrados en el apartado anterior de esta tesis han demostrado las propiedades insecticidas de dos de estas ribotoxinas (HtA, producida por *H. thompsonii*, y α-sarcina, producida por *A. giganteus*), y apoyan la posibilidad de que estén involucradas en los mecanismos de defensa del hongo frente a insectos. En esta revisión se repasan los aspectos de esta familia de proteínas que están relacionados con su toxicidad frente a insectos, haciendo hincapié en su relevancia biológica y planteando posibles aplicaciones biotecnológicas, como el desarrollo de biopesticidas alternativos a los métodos tradicionales del control de plagas.

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Fungal ribotoxins: Natural protein-based weapons against insects

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ABSTRACT

Ribotoxins are fungal extracellular ribonucleases highly toxic due to their ability to enter host cells and their effective ribonucleolytic activity against the ribosome. The natural role of these proteins in the producing fungi is still unsolved. Nevertheless, recent studies showing the insecticidal properties of two ribotoxins from different origin support their involvement in defense mechanisms. Thus, it seems that not just the entomopathogen *Hirsutella thompsonii* expresses the ribotoxin hirsutellin A as a virulence factor but also *Aspergillus*, the main ribotoxin producer, does so. In this review we focus on this little known aspect of this family of proteins, their toxicity against insects, from the point of view of its biological relevance and its potential biotechnological applications.

Nature provides numerous examples of self defense against predators in all living organisms. In particular, plants, fungi and bacteria produce a high variety of toxins against insects, many of which are proteins. From a biotechnological point of view, some of them are being developed for pest control, since conventional agrochemical pesticides show adverse effects on the environment and human health as well as a rapid emergence of pest resistance. Moreover, the high cost of discovering, developing and registering new synthetic pesticides have also contributed to increased interest in biopesticides (Glare et al., 2012).

Insect pathogenic fungi are of special interest since they play an important natural role in controlling insect pests. The approximately 1000 known species of entomopathogenic fungi target most, if not all, insect species, from sucking insects to many coleopteran and orthopteran pests, although individually have narrow host ranges. In fact, products based on the species *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria* are being commercialized (Faria and Wraight, 2007; Kim et al., 2014). However, efficacy limitations like dose response and specificity still need to be overcome. Therefore, a better understanding of fungal pathogenesis in insects at a molecular level is a demanding field in order to improve their application in a natural environment.

1. *Hirsutella thompsonii*

H. thompsonii is a well known entomopathogen fungus registered in 1981 as Mycar for the control of the citrus rust mite (McCoy, 1981). This formulation was soon abandoned due to its poor efficacy in field trials. Nevertheless, this mold has been recently studied against several mites. Mycohit is a formulation registered and commercialized in India for coconut eriophyid mites with success in extensive field trials studies (Sreerama Kumar and Singh, 2008). In fact, *Hirsutella* shows a high specificity to subclass Acari and it is clearly more virulent than the extensively studied

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Metarhizium anisopliae (Shaw et al., 2002; Rossi-Zalaf and Alves, 2006). *H. thompsonii* has been also tried as a biocontrol agent against the *Varroa* mite, the worst pathogen of the honey bee (Kanga et al., 2002; Peng et al., 2002). However, additional field assays are still to be performed. In addition, not much is known about the toxic molecules involved in its virulence. It has been described how extracellular media of a *H. thompsonii* culture has a pathogenic effect, with a very slow but high lethality against target insects (Vey et al., 1993). However, only the fungal ribotoxin hirsutellin A has been isolated and further studied, so far (Mazet and Vey, 1995).

2. Fungal ribotoxins

Extracellular chitinases and proteases are the protein-based toxins most abundant in insect pathogenic fungi, and are involved in cuticle invasion of their hosts. Nevertheless, they act in concert with many other factors (Clarkson and Charnley, 1996). Here we focus on a small but well established family of proteins named as fungal ribotoxins, which have potential virulence, although their biological role is still under study (Brandhorst and Kenealy, 1992; Yang and Kenealy, 1992). Ribotoxins are mainly produced by filamentous fungi of the genus *Aspergillus*, several of its species characterized as plant pathogens and in general a saprophytic genus (Martínez-Ruiz et al., 1999; Lacadena et al., 2007). The discovery of *H. thompsonii* as the only entomopathogen fungus producer of a ribotoxin, hirsutellin A, has opened a new view of these proteins as potential participants of an insecticidal activity (Herrero-Galán et al., 2008; Olombrada et al., 2013) (Fig. 1).

Fungal ribotoxins are extracellular ribonucleases with an extraordinary specificity against the ribosome. Their RNA target, the sarcin/ricin loop (SRL), is localized in the large ribosomal subunit and it is essential for ribosome function, participating in the activation step of several translation factors involved in all steps of protein synthesis (García-Ortega et al., 2010). Ribotoxins cleave just one phosphodiester bond of the SRL, enough to impair its essential function. This catalytic activity has been extensively studied as it has been their structure and relationship with other non-toxic extracellular ribonucleases also produced by fungi. In fact, there is an extended literature regarding these properties with the best known members of the family: α-sarcin, from *Aspergillus giganteus*, and restrictocin, from *Aspergillus restrictus* (Lacadena et al., 2007; Plantinga et al., 2008). These two fungal ribotoxins differ in just a 15% of their sequence showing practically identical structure and activity properties.

Due to their high efficacy to inactivate the ribosome of the target cell, fungal ribotoxins are considered one of the most potent toxins known. The universality of the SRL sequence and structure makes ribosomes from all kingdoms of life susceptible to ribotoxin action (Schindler and Davies, 1977; Endo and Wool, 1982; García-Ortega et al., 2010; Olombrada et al., 2013). Even the producer organism requires a high efficiency secretion pathway to prevent cytotoxicity (Lamy and Davies, 1991).

The toxicity of these proteins is also dependent on their ability to enter cells, with the limiting step based on lipid interaction (Oñaderra et al., 1993; Turnay et al., 1993; Olombrada et al., 2013). Preference for acidic phospholipids has been described, but a receptor interaction has not been

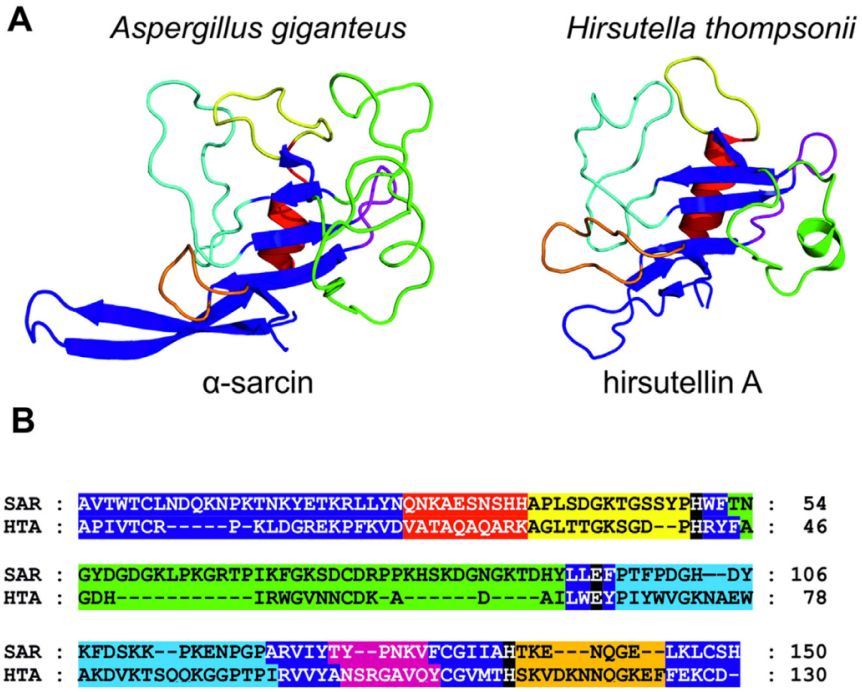

Fig. 1. Structure (A) and sequence alignment (B) of α-sarcin (PDB ID: 1DE3) and hirsutellin A (PDB ID: 2KAA). Color code of structural elements: β-sheet, dark blue; α-helix, red; loop 1, yellow; loop 2, green; loop 3, cyan; loop 4, magenta; loop 5, orange. Catalytic residues are black boxed in the sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

observed. Under laboratory conditions, fungal ribotoxins are able to penetrate into diverse mammal cells, such as several types of cancer- and virus-infected cells (Turnay et al., 1993). Their cytotoxicity is apparently not very specific, based on an endocytotic mechanism to reach the cytosol where they exert their toxic ribonucleolytic activity. However, these studies have been focused on their potential antitumoral effect. In fact, their antitumoral properties are being improved in terms of cell specificity by combining them with antibodies against certain cancer cells (Carreras-Sangrà et al., 2012). On the other hand, examples under natural conditions are scarce. Fungal ribotoxins share their target in the host cell, the ribosomal SRL, with the very well known ricin-like ribosome inactivating proteins (RIP), although they are unrelated proteins in terms of sequence, structure and enzymatic activity. RIPs constitute an extended family of N-glycosidases, mainly produced by plants that are classified into two main groups depending on their composition: one or two polypeptide chains (Stirpe and Battelli, 2006). Since many of them harbor a membrane receptor binding site, a lectin-like peptide, they show a higher toxicity than fungal ribotoxins with an improved mechanism to reach the intracellular space of host cells. In terms of their natural toxicity, RIPs have been shown to be toxic to coleopteran and lepidopteran insects (Gatehouse et al., 1990; Bertholdo-Vargas et al., 2009). However the high toxicity to mammals has invalidated them in their use as insecticides (Carlini and Grossi-de-Sá, 2002). Fungal ribotoxins could represent then a good alternative, encouraging their characterization in terms of natural toxicity.

3. Role in fungi development

There are just a few studies of fungal ribotoxin expression by their natural hosts. It was described how *A. restrictus* produces restrictocin at a very specific moment of development: the initial steps of conidiophore formation. In fact, restrictocin mRNA appears coincident with the protein, discarding its accumulation as an inactive form. In a water-stressed environment the ribotoxin is expressed at the surface of phialides in conidiophores. These results supported the idea of a survival advantage provided by these toxins based on a defense mechanism against predators like insects (Brandhorst and Kenealy, 1992; Yang and Kenealy, 1992). Moreover, *A. restrictus* is associated with some beetle species and it has been observed to have a deterrent effect on feeding by *Carpophilus freemani* correlated with the maximum restrictocin expression (Brandhorst et al., 1996). A specific role in conidia differentiation could also be possible for these ribotoxins, although further studies have not been performed.

In terms of pathogenesis in humans, fungal ribotoxins have been suggested to be responsible for inflammation in Aspergillosis infections (Arruda et al., 1990). However no detection of ribotoxin is observed in the initial steps of fungal infection but after cell mass accumulation, invalidating somehow an essential role in inflammation. Nevertheless, they do seem to play some role in allergic-like pathogenic processes like those ones leading to the establishment of aspergilloma (Crameri et al., 1998; Kurup et al., 1998; García-Ortega et al., 2005).

4. Insect toxicity

The fungal ribotoxin family consists of more than 20 members most of them made of about 150 amino acids and showing at least 85% sequence identity (Martínez-Ruiz et al., 1999). However, hirsutellin A is clearly different: smaller, with just a 35% sequence identity and with a different origin than the *Aspergillus* family since it is produced by the entomopathogen *H. thompsonii* (Lacadena et al., 2007; Herrero-Galán et al., 2008). Nevertheless its core structure is identical harboring a α-helix, a five-stranded antiparallel β-sheet and an N-terminal β-hairpin, differing from the rest of ribotoxins in the disordered loops surrounding it (Fig. 1). Its biochemical characterization has shown the same specific ribonucleolytic activity as α-sarcin, as well as a cytotoxic effect when assayed against eukaryotic cells (Herrero-Galán et al., 2008).

Hirsutellin A was previously described as toxic when injected in *Galleria mellonella* larvae and *per os* in neonatal larvae of *Aedes aegypti* (Mazet and Vey, 1995). These observations led to the proposal that it is a potential virulence factor of *H. thompsonii*, although further studies were never carried out. In fact, similar assays had not been performed with any other fungal ribotoxin until a recent study comparing hirsutellin A and α-sarcin where α-sarcin resulted nearly as toxic as hirsutellin A (Olombrada et al., 2013) (Fig. 2A). The idea of ribotoxins as insecticides and not just hirsutellin A as an exception was plausible. One fact supporting this idea is that *Aspergillus*, the main ribotoxin producer, shares feeding niche like the farm-stored grain with several insects, so competition for survival could have developed weapons like ribotoxins. The only example in literature was with restrictocin, tested in insect diet against *C. freemani* (beetle), *Spodoptera frugiperda* (caterpillar) and *Helicoverpa zea* (caterpillar) and showing a not very dramatic but significant toxic effect (Brandhorst et al., 1996). In agreement with the toxic effect of restrictocin, its heterologous expression in *Aspergillus nidulans*, a non ribotoxin producer, also inhibited insect feeding by *C. freemani* beetles (Brandhorst et al., 2001). Moreover, it was suggested a larger spectrum of insect toxicity for restrictocin than RIPs like, for example, ricin which has been shown innocuous to *S. frugiperda* larvae (Gatehouse et al., 1990; Brandhorst et al., 1996).

The biochemical characterization of the action of fungal ribotoxins in cultured insect cells showed how both hirsutellin A and α-sarcin are cytotoxic causing inhibition of protein synthesis upon reaching the intracellular space following penetration of the lipid bilayer (Olombrada et al., 2013) (Fig. 2B). Specific cleavage of ribosomal RNA in the insect ribosome agreed with all previous results with ribosomes from different origin (Lacadena et al., 2007; Herrero-Galán et al., 2008; García-Ortega et al., 2010). Here a dramatic difference was observed in terms of SRL cleavage efficiency and ribosome recognition, with hirsutellin A clearly more efficacious compared to α-sarcin (Fig. 2B). This result opens new approaches for ribotoxin-based development of insecticides. Since the comparison was performed just in a single model, *S. frugiperda* cells and ribosomes, an extensive study using several other

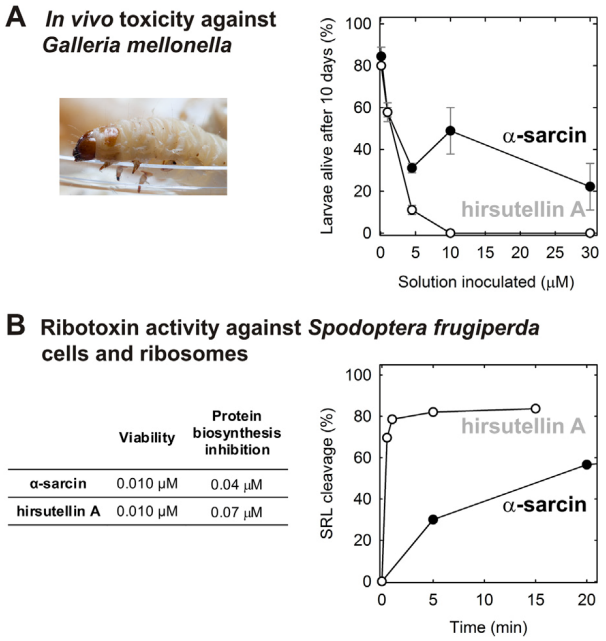

Fig. 2. (A) Toxicity of α-sarcin and hirsutellin A when injected in *Galleria mellonella* larvae. 8 μL of protein solution at different concentrations was inoculated in the hemocoel of 15 individuals per group. 0.9% NaCl was injected in the negative control. (B, table) IC_{50} of both ribotoxins in assays of viability and protein biosynthesis inhibition with Sf9 cultured cells. 3×10^5 cells were treated with different concentrations of ribotoxin for 60 h or 18 h respectively. Viability was measured by Trypan blue staining and protein biosynthesis by [^{3}H]-leucine incorporation after TCA precipitation. (B, graph) Kinetics data of specific cleavage of Sf9 isolated ribosomes for both ribotoxins at 50 nM concentration. Specific SRL cleavage was measured by primer extension analysis followed by denaturing electrophoresis separation and phosphorimager quantification. All data are adapted from Olombrada et al. (2013).

organisms could establish phyla-specificity. Both proteins were lethal when injected in *G. mellonella* larvae demonstrating a similar virulence potential (IC_{50} = 1.5 μM for hirsutellin A and 3.5 μM for α-sarcin) (Fig. 2A). How and when fungal ribotoxins have access to a living insect and how the producing fungi participate in this invasion are questions that still remain unanswered.

Finally, it has been observed that mechanisms of plant and insect infection by ascomycetes are similar (Sexton and Howlett, 2006), so it is also possible that fungal ribotoxins play the same role in a wide range defense mechanism worth against insects as is the case of the entomopathogen *H. thompsonii*, as well as against invading plants in several *Aspergillus* species.

5. Biotechnological applications

So far, trials with different fungi-based formula have been investigated for their use as biocontrol in agriculture. However, they present limitations in terms of activity spectra, delivery options and persistence (Glare et al., 2012). The discovery and study of isolated toxic molecules produced by pathogen microorganisms results in a better strategy, not only to understand their role in nature, but to combine and modify them in order to design improved biopesticides.

One strategy for biocontrol is the use of recombinant baculovirus as vectors to access the target pest species (Cory et al., 1994; Shim et al., 2013). This system has the advantage of being an easy and stable formula, as well as showing high specificity for the host. One example with a protein related to fungal pathogenesis consists of an improved baculovirus against *S. frugiperda* expressing a protease from *Aspergillus fumigatus* (Gramkow et al., 2010). In this case, either injected or fed, treated larvae showed an increased lethality when compared with those treated with the wild-type vector. These results encourage the development of similar tools with other fungal toxins, as it is the case of ribotoxins. Moreover, strategies like this one would make fungal ribotoxins insect-selective minimizing side toxic effects against other organisms.

The discovery and knowledge of fungal ribotoxins as potential virulence factors allow further research in the insecticide field with the aim to use them alone or combined with other biopesticides. Olombrada et al. (2013) focused on two ribotoxins, α-sarcin and hirsutellin A, produced by very different fungi, with important differences in terms of their efficacy to inactivate the ribosome and in their interaction with insect membranes, but similar as cytotoxins against insect cells and *G. mellonella* larvae (Fig. 2). These common and differential properties motivate additional comparative studies of their biological role, regarding insect specificity, effects in fertility, mechanism of administration and tolerance to the environment. As an example, differences in proteolytic susceptibility of fungal ribotoxins and RIPs by insect larvae have been suggested as one possible factor of differences in toxicity (Gatehouse et al., 1990; Brandhorst et al., 1996). This is the case of restrictocin that *H. zea* midgut homogenates were able to digest it 10 times faster than those of *S. frugiperda*, explaining in part the resistance of *H. zea* to the toxin (Brandhorst et al., 1996). Moreover, a wide variety of mutant versions of these fungal ribotoxins have been deeply characterized dissecting their functional properties in correlation with their structure. Examples are catalytically inactive variants of several ribotoxins (Lacadena et al., 2007); a variant of α-sarcin that lacking the N-terminal β-hairpin is catalytically active but unable to recognize the ribosome (García-Ortega et al., 2002) and a version of hirsutellin A without two Trp residues that is still active against the ribosome but unable to interact with lipids (Herrero-Galán et al., 2012). With all this molecular background the design of a more effective insecticidal toxin could be possible.

Finally, the inclusion of hisutellin A in the ribotoxin family suggests the idea of these proteins as a more widespread family among fungi. Moreover, the toxic effect of ribotoxins against insects reviewed here could be an example of their role in a more general defense mechanism in collaboration with other virulence factors. The understanding of their natural role in fungi defense as well as their improvement in terms of host specificity with tools like baculovirus vehicles are promising fields worth to keep focused on.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Arruda, L.K., Platts-Mills, T.A., Fox, J.W., Chapman, M.D., 1990. *Aspergillus fumigatus* allergen I, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. J. Exp. Med. 172, 1529–1532.

Bertholdo-Vargas, L.R., Martins, J.N., Bordin, D., Salvador, M., Schafer, A.E., Barros, N.M., Barbieri, L., Stirpe, F., Carlini, C.R., 2009. Type 1 ribosome-inactivating proteins – entomotoxic, oxidative and genotoxic action on *Anticarsia gemmatalis* (Hübner) and *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). J. Insect Physiol. 55, 51–58.

Brandhorst, T.T., Kenealy, W.R., 1992. Production and localization of restrictocin in *Aspergillus restrictus*. J. Gen. Microbiol. 138, 1429–1435.

Brandhorst, T., Dowd, P.F., Kenealy, W.R., 1996. The ribosome-inactivating protein restrictocin deters insect feeding on *Aspergillus restrictus*. Microbiology 142, 1551–1556.

Brandhorst, T., Dowd, P.F., Kenealy, W.R., 2001. The effect of fungal ribosome inactivating proteins upon feeding choice in *C. freemani*, and indications of a mutualistic relationship with *A. restrictus*. Mycopathologia 152, 155–158.

Carlini, C.R., Grossi-de-Sá, M.F., 2002. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon 40, 1515–1539.

Carreras-Sangrà, N., Tomé-Amat, J., García-Ortega, L., Batt, C.A., Oñaderra, M., Martínez-del-Pozo, A., Gavilanes, J.G., Lacadena, J., 2012. Production and characterization of a colon cancer-specific immunotoxin based on the fungal ribotoxin a-sarcin. Protein Eng. Des. Sel. 25, 425–435.

Clarkson, J.M., Charnley, A.K., 1996. New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol. 4, 197–203.

Cory, J.S., Hirst, M.L., Williams, T., Hails, R.S., Goulson, D., Green, B.M., Carty, T.M., Possee, R.D., Cayley, P.J., Bishop, D.H.L., 1994. Field trial of a genetically improved baculovirus insecticide. Nature 370, 138–140.

Crameri, R., Hemmann, S., Ismail, C., Menz, G., Blaser, K., 1998. Disease-specific recombinant allergens for the diagnosis of allergic bronchopulmonary aspergillosis. Int. Immunol. 10, 1211–1216.

Endo, Y., Wool, I.G., 1982. The site of action of α-sarcin on eukaryotic ribosomes. The sequence at the α-sarcin cleavage site in 28S ribosomal ribonucleic acid. J. Biol. Chem. 257, 9054–9060.

Faria, M.R., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control 43, 237–256.

García-Ortega, L., Alvarez-García, E., Gavilanes, J.G., Martínez-del-Pozo, A., Joseph, S., 2010. Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. Nucleic Acids Res. 38, 4108–4119.

García-Ortega, L., Lacadena, J., Villalba, M., Rodríguez, R., Crespo, J.F., Rodríguez, J., Pascual, C., Olmo, N., Oñaderra, M., Martínez-del-Pozo, A.M., Gavilanes, J.G., 2005. Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Aspf1 displaying reduced IgE binding. FEBS J. 272, 2536–2544.

García-Ortega, L., Masip, M., Mancheño, J.M., Oñaderra, M., Lizarbe, M.A., García-Mayoral, M.F., Bruix, M., Martínez del Pozo, A., Gavilanes, J.G., 2002. Deletion of the NH2-terminal beta-hairpin of the ribotoxin alpha-sarcin produces a nontoxic but active ribonuclease. J. Biol. Chem. 277, 18632–18639.

Gatehouse, A.M.R., Barbieri, L., Stirpe, F., Croy, R.R.D., 1990. Effects of ribosome inactivating proteins on insect development. Differences between Lepidoptera and Coleoptera. Entomol. Exp. Appl. 54, 43–51.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Köhl, J., Marrone, P., Morin, L., Stewart, A., 2012. Have biopesticides come to age? Trends Biotechnol. 30, 250–258.

Gramkow, A.W., Perecmanis, S., Sousa, R.L., Noronha, E.F., Felix, C.R., Nagata, T., Ribeiro, B.M., 2010. Insecticidal activity of two proteases against *Spodoptera frugiperda* larvae infected with recombinant baculoviruses. Virol. J. 7, 143. http://dx.doi.org/10.1186/1743-422X-7-143.

Herrero-Galán, E., García-Ortega, L., Lacadena, J., Martínez-del-Pozo, Á., Olmo, N., Gavilanes, J.G., Oñaderra, M., 2012. A non-cytotoxic but ribonucleolytically specific ribotoxin variant: implication of tryptophan residues in the cytotoxicity of hirsutellin A. Biol. Chem. 393, 449–456.

Herrero-Galán, E., Lacadena, J., Martínez-del-Pozo, A., Boucias, D.G., Olmo, N., Oñaderra, M., Gavilanes, J.G., 2008. The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. Proteins 72, 217–228.

Kanga, L.H., James, R.R., Boucias, D.G., 2002. *Hirsutella thompsonii* and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor*, a honey bee parasite. J. Invertebr. Pathol. 81, 175–184.

Kim, C.S., Lee, J.B., Kim, B.S., Shin, K.S., Kim, J.W., Kwon, G.S., 2014. A Technique for the prevention of Greenhouse whitefly (*Trialeurodes vaporariorum*) by using Entomopathogenic Fungi *Beauveria bassiana* M130. J. Microbiol. Biotechnol. 24, 1–7.

Kurup, V.P., Banerjee, B., Murali, P.S., Greenberger, P.A., Krishnan, M., Hari, V., Fink, J.N., 1998. Immunodominant peptide epitopes of allergen, Asp f 1 from the fungus *Aspergillus fumigatus*. Peptides 19, 1469–1477.

Lacadena, J., Alvarez-García, E., Carreras-Sangrà, N., Herrero-Galán, E., Alegre-Cebollada, J., García-Ortega, L., Oñaderra, M., Gavilanes, J.G., Martínez-del-Pozo, A., 2007. Fungal ribotoxins: molecular dissection of a family of natural killers. FEMS Microbiol. Rev. 31, 212–237.

Lamy, B., Davies, J., 1991. Isolation and nucleotide sequence of the *Aspergillus restrictus* gene coding for the ribonucleolytic toxin restrictocin and its expression in *Aspergillus nidulans*: the leader sequence protects producing strains from suicide. Nucleic Acids Res. 19, 1001–1006.

Martínez-Ruiz, A., Kao, R., Davies, J., Martínez-del-Pozo, A., 1999. Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. Toxicon 37, 1549–1563.

Mazet, I., Vey, A., 1995. Hirsutellin A, a toxic protein produced *in vitro* by *Hirsutella thompsonii*. Microbiology 141, 1343–1348.

McCoy, C.W., 1981. Pest control by the fungus *Hirsutella thompsonii*. In: Burges, H.D., Hussey, N.W. (Eds.), Microbial Control of Pests and Plant Diseases 1979–1980. Academic Press, New York, pp. 499–512.

Olombrada, M., Herrero-Galán, E., Tello, D., Oñaderra, M., Gavilanes, J.G., Martínez-del-Pozo, Á., García-Ortega, L., 2013. Fungal extracellular ribotoxins as insecticidal agents. Insect Biochem. Mol. Biol. 43, 39–46.

Oñaderra, M., Mancheño, J.M., Gasset, M., Lacadena, J., Schiavo, G., Martínez-del-Pozo, A., Gavilanes, J.G., 1993. Translocation of alpha-sarcin across the lipid bilayer of asolectin vesicles. Biochem. J. 295, 221–225.

Peng, C.Y., Zhou, X., Kaya, H.K., 2002. Virulence and site of infection of the fungus, *Hirsutella thompsonii*, to the honey bee ectoparasitic mite, *Varroa destructor*. J. Invertebr. Pathol. 81, 185–195.

Plantinga, M.J., Korennykh, A.V., Piccirilli, J.A., Correll, C.C., 2008. Electrostatic interactions guide the active site face of a structure-specific ribonuclease to its RNA substrate. Biochemistry 47, 8912–8918.

Rossi-Zalaf, L.S., Alves, S.B., 2006. Susceptibility of *Brevipalpus phoenicis* to entomopathogenic fungi. Exp. Appl. Acarol. 40, 37–47.

Schindler, D.G., Davies, J.E., 1977. Specific cleavage of ribosomal RNA caused by alpha sarcin. Nucleic Acids Res. 4, 1097–1110.

Sexton, A.C., Howlett, B.J., 2006. Parallels in fungal pathogenesis on plant and animal hosts. Eukaryot. Cell 5, 1941–1949.

Shaw, K.E., Davidson, G., Clark, S.J., Ball, B.V., Pell, J.K., Chandler, D., Sunderland, K.D., 2002. Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata) an ectoparasite mite of the honeybee, *Apis mellifera*. Biol. Control 24, 266–276.

Shim, H.J., Choi, J.Y., Wang, Y., Tao, X.Y., Liu, Q., Roh, J.Y., Kim, J.S., Kim, W.J., Woo, S.D., Jin, B.R., Je, Y.H., 2013. NeuroBactrus, a novel, highly effective and environmentally friendly recombinant baculovirus insecticide. Appl. Environ. Microbiol. 79, 141–149.

Sreerama Kumar, P., Singh, L., 2008. Enabling mycelial application of *Hirsutella thompsonii* for managing the coconut mite. Exp. Appl. Acarol. 46, 169–182.

Stirpe, F., Battelli, M.G., 2006. Ribosome-inactivating proteins: progress and problems. Cell Mol. Life Sci. 63, 1850–1866.
Turnay, J., Olmo, N., Jiménez, A., Lizarbe, M.A., Gavilanes, J.G., 1993. Kinetic study of the cytotoxic effect of alpha-sarcin, a ribosome inactivating protein from *Aspergillus giganteus*, on tumour cell lines: protein biosynthesis inhibition and cell binding. Mol. Cell. Biochem. 122, 39–47.
Vey, A., Quiot, J.M., Mazet, I., McCoy, C.W., 1993. Toxicity and pathology of crude broth filtrate produced by *Hirsutella thompsonii* var. *thompsonii* in shake culture. J. Invertebr. Pathol. 61, 131–137.
Yang, R., Kenealy, W.R., 1992. Regulation of restrictocin production in *Aspergillus restrictus*. J. Gen. Microbiol. 138, 1421–1427.

Resultados C: EL RIBOSOMA EUCARIOTA COMO DIANA DE LAS RIBOTOXINAS.

C1. El tallo ribosómico no participa en la inactivación específica ejercida por la α-sarcina sobre el ribosoma eucariota

Las ribotoxinas fúngicas son una familia de RNasas capaces de cortar un único enlace fosfodiéster en una secuencia conservada del rRNA, el lazo sarcina/ricina (SRL), lo que afecta al proceso de traducción e inhibe la síntesis de proteínas. Las propiedades enzimáticas de las ribotoxinas se conocen en detalle, aunque el mecanismo molecular por el que estas toxinas reconocen el SRL apenas se ha investigado. Las proteínas ácidas P0, P1 y P2 del tallo ribosómico se localizan cerca de la región del ribosoma implicada en la unión de los factores de elongación al ribosoma. Recientemente se ha demostrado la participación del tallo ribosómico eucariota en el mecanismo de acción de otra proteína inactivante del ribosoma, la ricina. En este trabajo se ha investigado el papel que juega esta estructura del ribosoma en el mecanismo de acción de la ribotoxina α-sarcina. Experimentos con ribosomas aislados, sistemas de traducción libres de células y ensayos de viabilidad en distintas cepas mutantes de la levadura *Saccharomyces cerevisiae,* con diferente composición del tallo ribosómico, han permitido demostrar que, al contrario de lo que sucede con la ricina y otras proteínas inactivantes del ribosoma, el tallo ribosómico no parece estar involucrado en la actividad ribonucleolítica específica de la α-sarcina frente al ribosoma eucariota. Por tanto, a pesar de compartir la misma diana en el ribosoma, parece que la ricina y la α-sarcina utilizan mecanismos diferentes de interacción con el ribosoma eucariota.

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The Acidic Ribosomal Stalk Proteins Are Not Required for the Highly Specific Inactivation Exerted by α-Sarcin of the Eukaryotic Ribosome

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Supporting Information

ABSTRACT: The ribosomal sarcin/ricin loop (SRL) is the target of ribosome-inactivating proteins like the *N*-glycosidase ricin and the fungal ribotoxin α-sarcin. The eukaryotic ribosomal stalk directly interacts with several members of the *N*-glycosidase family, favoring their disruption of the SRL. Here we tested this hypothesis for the ribotoxin α-sarcin. Experiments with isolated ribosomes, cell-free translation systems, and viability assays with *Saccharomyces cerevisiae* strains defective in acidic stalk proteins showed that the inactivation exerted by α-sarcin is independent of the composition of the ribosomal stalk. Therefore, α-sarcin, with the same ribosomal target as ricin, seems to access the SRL by a different pathway.

Ribosome-inactivating proteins have been extensively studied because of their extraordinary efficiency against their target cells. They are classified into two groups: those with *N*-glycosidase activity (RIPs), like ricin,[1] and ribotoxins, a family of fungal ribonucleases best represented by α-sarcin.[2] Considering their unrelated sequence, structure, and catalytic activity, it is striking how they recognize the same element in the ribosome, the sarcin/ricin loop (SRL), essential for translation factor binding and their GTPase activation.[3,4] Despite the universality of the SRL, ribotoxins and RIPs show important differences in their efficiencies against ribosomes of different origins.[1] Most of them prefer eukaryotic ribosomes, suggesting a specific recognition of additional elements in their structure.[5,6] Moreover, it has been shown how the ribosomal stalk can be used as an important interacting element for RIPs like ricin, trichosanthin, and Shiga-like toxins.[7,8] This protruding structure in the ribosome has been described as being involved in the recruitment and turnover of elongation factors during translation.[9,10]

The composition of the stalk is fairly similar among organisms, although important differences in the sequences of the corresponding proteins have been identified[11−13] (Figure S1 of the Supporting Information). It is based on a central protein, P0 in eukaryotes (L10 in prokaryotes), and dimers of acidic proteins bound to it (P1 and P2 in eukaryotes and L7/12 in prokaryotes). In particular, *Saccharomyces cerevisiae* displays two heterodimers formed by P1 and P2 isoforms: P1αP2β and P1βP2α. These acidic proteins are extremely dynamic, including their exchange with the cytoplasmic pool, their C-terminal regions being responsible for the interaction, recruitment, and regulation of supernatant translation factors and ribosome-inactivating proteins like ricin and trichosanthin.[9,14] However, nothing is known about ribotoxins in this regard.

Ribotoxins make up an interesting family of basic ribonucleases secreted by filamentous fungi.[2] They harbor in their small structure the ability to penetrate into target cells by interacting with membrane lipids and their extraordinary efficiency against the SRL in the ribosome. α-Sarcin is the most representative member of the family and has been exhaustively characterized. However, the mechanism of ribosome recognition is still far from being well understood. On the basis of detailed kinetic analyses, it was shown how the electrostatic character of the ribosomal surface promotes a rapid guiding of ribotoxins toward their target, a concept that was later extended to several other RIPs.[15]

Considering the role of the P1 and P2 proteins of the eukaryotic ribosomal stalk in the positioning of several RIPs to the SRL,[7,8,14,16−18] we have studied the potential interaction of ribotoxins with them. Unexpectedly, the results obtained suggest a different mode of action for fungal ribotoxins.

To first study the role of the ribosomal stalk in the specific recognition and cleavage of the SRL by α-sarcin, purified ribosomes with (wild type) and without P1 and P2 proteins (ΔP1P2) were chosen (Figure S1 of the Supporting Information). Quantification of this cleavage by primer extension analysis gave very similar results in both cases (Figure 1A). Considering that salt concentration is critical for ribosome stability and functionality, as well as the effect of electrostatics in its interaction with ribotoxins and RIPs,[15,18] the effect of changes in Mg^{2+} concentration was tested. The increment from 1 to 5 mM Mg^{2+} made both types of ribosomes less accessible to α-sarcin, as expected, but no differences were observed between them (Figure 1A). Interestingly, the characteristic ribonucleolytic activity of α-sarcin on ribosomes was higher when α-sarcin was assayed in the presence of additional translating factors (by including a ΔP1P2 S100 extract), but substantial differences were not found between wild-type (WT) and mutant ribosomes (Figure 1B). Finally, it was also demonstrated that free cytosolic

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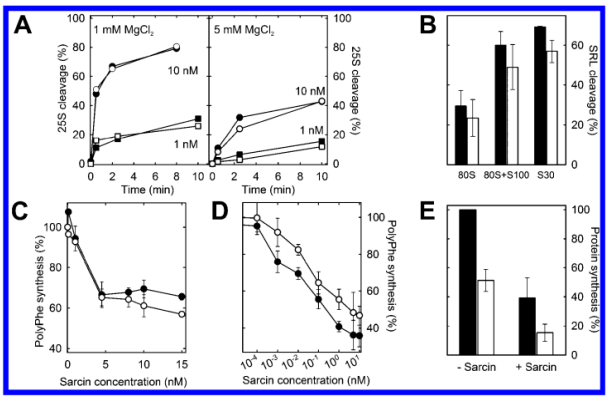

Figure 1. (A) *In vitro* SRL cleavage of ribosomes from WT and ΔP1P2 yeast strains treated with α-sarcin. (B) α-Sarcin (10 nM) against isolated ribosomes in the presence of polyU (80S), supplemented with ΔP1P2 S100 (80S+S100), or against S30 lysates. (C) Effect of α-sarcin in polyPhe synthesis by WT or ΔP1P2 ribosomes supplemented with ΔP1P2 S100. PolyPhe was quantified by precipitating [^{3}H]Phe peptides. (D) Identical experiment as in panel C but with WT or ΔP1P2 S30 extracts. (E) Endogenous mRNA translation by WT or ΔP1P2 S30 extracts in the presence (+) or absence (−) of α-sarcin. Protein synthesis was quantified by the amount of [^{35}S]Met incorporated. Black symbols and bars are used for WT and white symbols and bars for ΔP1P2.

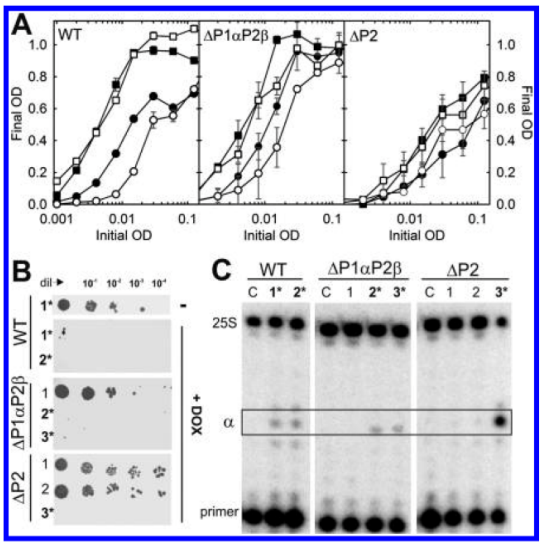

Figure 2. (A) Effect of WT (circles) and H50/137Q/E96Q (squares) α-sarcin expression in WT, ΔP1α*P2β*, and ΔP2 *S. cerevisiae* strains. Growth curves in the presence (white) or absence (black) of doxycycline. (B) Viability assay spotting serial dilutions of pCM175α*S* transformed strains in SC-W without (−) and with (+DOX) doxycycline. Several clones are shown, and those marked with asterisks are sensitive to α-sarcin toxicity. (C) Primer extension analysis of RNA extracted from liquid cultures of clones shown in panel B. Clones transformed with an empty control vector (C) were included. Bands corresponding to intact 25S, α-sarcin specific cleavage (α), and an excess of primer are indicated.

P proteins do not have any impact in the SRL cleavage by α-sarcin by using S30 extracts in the assay (Figure 1B).

Measurement of the effect of ribotoxins on ribosome function is more sensitive than direct quantification of SRL cleavage. Moreover, as ribosomes defective in stalk proteins are less efficient in translation, a similar extent of SRL cleavage by ribotoxins may be more efficient in terms of the inhibition of protein synthesis. Therefore, different cell-free translation systems were designed and assayed for α-sarcin inhibition. System A included isolated WT and ΔP1P2 ribosomes and ΔP1P2 S100 extract to translate polyU into polyPhe (Figure 1C). This assay measures the effect of the absence of the stalk proteins. System B includes a more efficient system of polyPhe synthesis using WT and ΔP1P2 S30 lysates, where the cytosolic P protein pool is also a variable (an important fact considering that it has been postulated for ricin that cytosolic P proteins help in the recruitment of these RIPs to the SRL binding site[7]) (Figure 1D). In system C, the translation of the endogenous mRNAs included in the S30 lysates was also assayed for the action of α-sarcin, where steps like initiation are taken into account (Figure 1E). Results from these experiments showed that the absence of P proteins did not affect the inhibitory action of α-sarcin against translating ribosomes when tested in cell-free systems.

To perform an *in vivo* analysis, doxycycline inducible intracellular expression of WT α-sarcin was accomplished in *S. cerevisiae* W303 cells using a catalytically inactive mutant as a negative control (α-sarcin H50/137Q/E96Q, sar3M). Toxicity was analyzed by means of cell growth impairment that, for the WT protein, was observed even in the absence of induction, in agreement with the existence of a minimal, but not negligible, basal expression in this system[19] (Figure 2A). This result is especially remarkable considering that how the intracellular presence of just one single molecule of α-sarcin can be lethal has been described and agrees with previously published data.[20] The specific SRL cleavage on host ribosomes by α-sarcin was confirmed to discard other side effects as a cause of the observed toxicity (Figure S2 of the Supporting Information). The production of α-sarcin was also studied in two *S. cerevisiae* strains defective in the acidic P proteins: ΔP1α*P2β* (lacking one heterodimer) and ΔP2 (lacking both heterodimers) (Figure S1 of the Supporting Information). Growth curves showed smaller differences when WT ribotoxin was expressed in the ΔP1α*P2β* strain, and not a considerable defect in growth was detected in the strain ΔP2 (Figure 2A). Because the *in vitro* results suggested that the ribosomal stalk did not directly participate in the ribotoxin inactivation of the ribosome, it seemed that the slower-growing defective strains would favor the appearance of ribotoxin resistance. To further analyze this idea, six individual clones of each strain were tested in solid medium viability assays (Figure 2B). In accordance, this assay showed that α-sarcin was lethal in all WT clones, while 30 and 80% of resistant clones were obtained for the ΔP1α*P2β* and ΔP2 strains, respectively.

Interpretation of these results in terms of the involvement of the stalk proteins in α-sarcin toxicity required the analysis of the ribotoxin resistance in ΔP1α*P2β* and ΔP2 strains. Ribosomal RNA was analyzed for all clones, and the α-sarcin cleavage band was present only in those cases where there was a toxic effect inhibiting yeast growth (Figure 2C). Therefore, α-sarcin specific ribonucleolytic activity was always linked to the lethal phenotype of the hosting clones. Finally, all clones were subjected to α-sarcin cDNA amplification analyses. Surprisingly, only those clones for which α-sarcin showed a lethal phenotype in agar plates maintained its cDNA (Figure S3 of the Supporting Information). The difference between WT and stalk defective strains in maintaining the ribotoxin gene may be due to additional factors not involving a direct interaction between the ribosomal stalk and α-sarcin. For example, P protein knockout strains show defective growth, and their pattern of protein

expression changes.[21] Consequently, resistance to α-sarcin expression did not arise from an incomplete ribosomal stalk but rather from a selection pressure to delete the α-sarcin gene.

This study focuses on the ribosomal stalk, involved in recruiting and activating different translation factors during protein synthesis.[9,10,12] As mentioned above, it also serves as an anchoring platform for several RIPs like ricin and trichosantin to further recognize their target, the SRL.[7,8,14,16−18] This interaction explains the specificity of these toxins for eukaryotic ribosomes, based on their different sequences with respect to their bacterial counterparts. However, not all RIPs behave identically. For example, for the pokeweed antiviral protein, no interaction with the ribosomal stalk has been found.[22] Along those same lines, and in agreement with the results shown here, a recent study has shown that the P1/P2 C-terminal peptide SDDDMGFGLFD does not interact with α-sarcin (a ribotoxin) or saporin (a RIP) but does interact with Shiga-like toxin A1.[23] These differences have been explained in terms of their different surface charge distributions. In the case of ricin, positively charged arginine residues on the opposite side of the active center seem to be essential for its interaction with the stalk.[18] Overall, this heterogeneity has led to the suggestion that evolution of RIPs has been recent and convergent, with the aim of interacting with the ribosomal stalk.[24]

Here we focus on fungal ribotoxins.[2] The idea of the stalk participating in helping α-sarcin reach the SRL was plausible considering previous observations for ricin. First, α-sarcin shares an identical target with ricin despite being a completely different enzyme. Second, the strong basic character of α-sarcin suggests it as a good candidate to interact with the acidic proteins of the stalk. Moreover, as for ricin, it is generally accepted that α-sarcin is more active against eukaryotic ribosomes, although an extensive comparison has not been performed.[5] Finally, interactions with stalk proteins do not seem to be highly selective in terms of docking specific structures, because models with trichosanthin and eEF2 show how these two very different proteins are able to interact with overlapping regions of P proteins.[16] However, our results show that the acidic ribosomal stalk P proteins do not participate in α-sarcin inactivation of the ribosome. The exhaustive *in vitro* characterization performed has not been able to reveal any influence of these proteins on the action of α-sarcin (Figure 1). Furthermore, *in vivo* experiments have shown the extraordinary toxicity of α-sarcin when it is expressed in yeast, which is independent of the composition of the stalk (Figure 2).

In conclusion, this study reveals that a fully assembled ribosomal stalk does not favor the ribotoxin specific cleavage of the yeast ribosome as opposed to the observations reported for other *N*-glycosidases like ricin, trichosantin, or Shiga-like toxins.[7,8,14] In addition, *in vitro* translation inhibition experiments suggest a preferred action of α-sarcin against translating ribosomes (Figure 1B). Therefore, the molecular basis for ribotoxin specific recognition of the SRL within the ribosome is still to be discovered.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

■ DEDICATION

‖This article is dedicated to the memory of María Rodríguez-Mateos, who passed away while this study was being performed.

■ REFERENCES

(1) Stirpe, F., and Battelli, M. G. (2006) *Cell. Mol. Life Sci. 63*, 1850−1866.
(2) Lacadena, J., Álvarez-García, E., Carreras-Sangrà, N., Herrero-Galán, E., Alegre-Cebollada, J., García-Ortega, L., Oñaderra, M., Gavilanes, J. G., and Martínez-del-Pozo, A. (2007) *FEMS Microbiol. Rev. 31*, 212−237.
(3) García-Ortega, L., Álvarez-García, E., Gavilanes, J. G., Martínez-del-Pozo, A., and Joseph, S. (2010) *Nucleic Acids Res. 38*, 4108−4119.
(4) Voorhees, R. M., Schmeing, T. M., Kelley, A. C., and Ramakrishnan, V. (2010) *Science 330*, 835−838.
(5) Schindler, D. G., and Davies, J. E. (1977) *Nucleic Acids Res. 4*, 1097−1110.
(6) Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987) *J. Biol. Chem. 262*, 5908−5912.
(7) Chiou, J. C., Li, X. P., Remacha, M., Ballesta, J. P., and Tumer, N. E. (2008) *Mol. Microbiol. 70*, 1441−1452.
(8) Tumer, N. E., and Li, X. P. (2012) *Curr. Top. Microbiol. Immunol. 357*, 1−18.
(9) Helgstrand, M., Mandava, C. S., Mulder, F. A., Liljas, A., Sanyal, S., and Akke, M. (2007) *J. Mol. Biol. 365*, 468−479.
(10) Mochizuki, M., Kitamyo, M., Miyoshi, T., Ito, K., and Uchiumi, T. (2012) *Genes Cells 17*, 273−284.
(11) Gonzalo, P., and Reboud, J. P. (2003) *Biol. Cell 95*, 179−193.
(12) Diaconu, M., Kothe, U., Schünzen, F., Fischer, N., Hams, J. M., Tonevitsky, A. G., Stark, H., Rodnina, M. V., and Wahl, M. C. (2005) *Cell 121*, 991−1004.
(13) Lee, K. M., Yu, C. W., Chiu, T. Y., Sze, K. H., Shaw, P. C., and Wong, K. B. (2012) *Nucleic Acids Res. 40*, 3172−3182.
(14) Lee, K. M., Yusa, K., Chu, L. O., Yu, C. W., Oono, M., Miyoshi, T., Ito, K., Shaw, P. C., Wong, K. B., and Uchiumi, T. (2013) *Nucleic Acids Res. 41*, 8776−8787.
(15) Korennykh, A. V., Correll, C. C., and Piccirilli, J. A. (2007) *RNA 13*, 1391−1396.
(16) Chan, D. S., Chu, L. O., Lee, K. M., Too, P. H., Ma, K. W., Sze, K. H., Zhu, G., Shaw, P. C., and Wong, K. B. (2007) *Nucleic Acids Res. 35*, 1660−1672.
(17) Li, X. P., Grela, P., Krokowsi, D., Tchorzewski, M., and Tumer, N. E. (2010) *J. Biol. Chem. 285*, 41463−41471.
(18) Li, X. P., Kahn, P. C., Kahn, J. N., Grela, P., and Tumer, N. E. (2013) *J. Biol. Chem. 288*, 30270−30284.
(19) Garí, E., Piedrafita, L., Aldea, M., and Herrero, E. (1997) *Yeast 13*, 837−848.
(20) Yang, R., and Kenealy, W. R. (1992) *J. Biol. Chem. 267*, 16801−16805.
(21) Cárdenas, D., Revuelta-Cervantes, J., Jiménez-Díaz, A., Camargo, H., Remacha, M., and Ballesta, J. P. (2012) *Nucleic Acids Res. 40*, 4520−4529.
(22) Ayub, M. J., Smulski, C. R., Ma, K. W., Levin, M. J., Shaw, P. C., and Wong, K. B. (2008) *Biochem. Biophys. Res. Commun. 369*, 314−319.
(23) McCluskey, A. J., Bolewska-Pedyczak, E., Jarvik, N., Chen, G., Sidhu, S. S., and Gariépy, J. (2012) *PLoS One 7*, e31191.
(24) Lapadula, W. J., Sanchez-Puerta, M. V., and Ayub, M. J. (2012) *Toxicon 59*, 427−432.

EXPERIMENTAL PROCEDURES

Escherichia coli* and *Saccharomyces cerevisiae* strains, vectors and ribotoxins.** *E. coli* DH5α and XL1-Blue strains were used for plasmid manipulations. Yeast strains used in this study were *S. cerevisiae* W303 (*MATa**, *ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100)*, and derived mutants D45 (lacking P2α and P2β, designated as ΔP2 from now on), D57 (lacking P1α and P2β; named as ΔP1αP2β from now on) and D4567 (lacking the four P proteins and referred as ΔP1P2 from now on) (1, 2) (Figure S1). Except for P0, none of the other stalk acidic proteins is essential for *Saccharomyces*; however, growth was notably impaired when one, and especially when both dimers were deleted.

The study was performed with wild-type α-sarcin and two mutant variants: a catalytically inactive mutant, α-sarcin H50/137Q/E96Q (3), and a deletion version, α-sarcin [Δ(7-22)] (4), active but unable to specifically cleave the SRL. Their cDNA were cloned in the doxycycline inducible vectors pCM175 and pCM252 (5, 6). All these constructs were confirmed by sequencing at the DNA Sequencing Unit of Parque Científico de Madrid. In addition, wild-type fungal natural α-sarcin was also produced from the mold *Aspergillus giganteus* and purified as previously described (7).

***S. cerevisiae* ribosomes, S100 and S30 purification.** Wild-type and ΔP1P2 ribosomes were isolated from 2 liter cultures of W303 and D4567 strains, respectively, grown up to OD_{600}= 0.5. The procedure followed was that described (8) with some modifications. Once cells were recovered, they were resuspended in 20 mL of T1 buffer (20 mM Tris-HCl, pH 7.5, 80 mM KCl, 12.5 mM $MgCl_2$, 5 mM β-mercaptoethanol) supplemented with 0.2 mM PMSF and a proteases inhibitor cocktail. Cell lysis was

performed with two cycles in a French press at 18.000 psi. The cell lysate, once cleared, was centrifuged at 115000 g for 2h at 4 °C. The resulting supernatant was the S100 fraction, containing cytosolic proteins including factors required for translation and cytosolic P proteins, when not deleted from the genome. This was concentrated to 5 mL in YM-3 Centriprep and fractioned using a differential precipitation with ammonium sulfate (8). Precipitated fraction was resuspended in 1 mL of 50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol and stored in aliquots at -80ºC.

The pellet of crude ribosomes, was resuspended in 150 μL of T2 buffer (20 mM Tris-HCl, pH 7.4, 500 mM NH_4Ac, 100 mM $MgCl_2$, 5 mM β-mercaptoethanol), also supplemented with 0.2 mM PMSF and a proteases inhibitor cocktail, and centrifuged through a discontinuous sucrose gradient (20%/40%) in T2 at 115000 g for 18h at 4°C. The new pellet of washed ribosomes was dissolved in a small volume of T1, aliquoted and stored at –80 °C.

Wild-type and ΔP1P2 cells lysates (the S30 fraction) were obtained following Henderson and Hershey's protocol (9). The cell pellet from 2L culture was now washed twice with 5 mL of T3 buffer (30 mM HEPES pH 7.4, 100 mM KAc, 2 mM $MgCl_2$, 2 mM DTT) containing 50 μM amino acids and 100 U/mL RNasin and then finally resuspended in 0.5 mL of this T3 buffer. Lysis was performed with several cycles of vortexing in the presence of glass beads. Then, this lysate was clarified by centrifugation and desalted through a PD10 column (GE Healthcare) equilibrated in T3. Final S30 pool was adjusted to the mentioned amino acids and RNasin concentrations and stored at -80ºC in small aliquots.

α-Sarcin ribonucleolytic activity assay. The specific and unique ribonucleolytic action of ribotoxins on ribosomes results in the cleavage of a single phosphodiester bond located at the SRL with the subsequent release of a characteristic rRNA fragment (the α-fragment). α-Sarcin was assayed against isolated ribosomes in 30 mM HEPES, pH 7.5, 50 mM KCl, 1 or 5 mM $MgCl_2$, 5 mM β-mercaptoethanol at 30ºC. Ribosome concentration was 0.1-0.4 μM. Incubation times varied from 30 sec to 30 min and ribotoxin concentrations from 0.1 to 200 nM. The reaction was stopped with 0.3 M NaAc, 1% (w/v) SDS, the RNA was phenol/chloroform extracted and finally precipitated with ethanol. The RNA obtained was analyzed by poison primer extension as described before (10). With this purpose, reverse transcription where dATP is substituted by ddATP was performed using the primer 5'-ACCAATTATCCGAATGAACTG-3' that complements the sequence downstream the SRL in 25S rRNA of *S. cerevisiae*. The products of reverse transcription were then separated in a 10% denaturing polyacrylamide gel and quantified using a PhosphorImager (Molecular Dynamics). Results were expressed as percentage of SRL cleavage. RNA extraction and poison primer extension was also employed to analyze the effect of α-sarcin on different cell-free translation systems described below.

Ribosomal RNA from *S. cerevisiae* strains expressing the different ribotoxins was also isolated to quantify α-sarcin cleavage. With this aim, cell pellets from 5 mL cultures at 0.3-1.0 OD_{600} values were resuspended in 100 μL of breaking buffer (50 mM HEPES-HCl, pH 7.5, 1 mM EDTA, 5% glycerol, 1 mM PMSF). Lysis was performed by vortexing in glass beads and RNA extraction was made as described above.

***In vitro* protein synthesis inhibition.** Three different assays were used to study α-sarcin inactivation of ribosome protein synthesis: Polyphenylalanine (polyPhe) synthesis with WT and ΔP1P2 ribosomes supplemented with ΔP1P2 S100 (to avoid involvement of free cytosolic P proteins); polyPhe synthesis using WT and ΔP1P2 S30 lysates, and translation of endogenous mRNA using WT and ΔP1P2 S30 lysates in order to include the step of initiation.

PolyPhe synthesis by purified WT and ΔP1P2 ribosomes supplemented with ΔP1P2 S100 was made in a final volume of 25 μL of 30 mM HEPES-HCl, pH 7.5, 50 mM KCl, 5 $MgCl_2$ and 2 mM β-mercaptoethanol, containing 0.1 mg/mL of $tRNA^{Phe}$ (from brewer's yeast, Sigma), 40 μM Phenylalanine, 0.5 μCi L-[4-^{3}H]-Phenylalanine, 60 μg/mL polyuridylic acid (polyU), 0.5 mM GTP, 1 mM ATP, 4 mM creatine phosphate and 25 μg/mL creatine kinase (8). Ribosome concentration was 0.1 μM and S100 presence was minimized to the smallest amount (1-3 μL). Ribotoxin concentrations ranged from 0.1 to 20 nM. Samples were incubated for 30 min at 30ºC and then stopped with 1 mL of 5% TCA. Protein was precipitated by 15 min incubation at 90ºC and radioactivity was measured in a Beckman LS 3801 liquid scintillation counter.

PolyPhe synthesis by S30 lysates was performed following the same procedure described above. The amount of S30 from WT or ΔP1P2 *S. cerevisiae* strains was optimized to obtain the maximum of polyPhe synthesis, but it was never higher than 10% of the final volume. A 0.1 pM to 0.1 μM range of α-sarcin concentrations was assayed.

Since ΔP1P2 ribosomes are less efficient than intact ribosomes in protein translation (1, 2) results of PolyPhe synthesis were separately normalized considering 100% synthesis that value without α-sarcin in each case.

The endogenous mRNA translation assay was performed in 25 μL of 30 mM HEPES-HCl, pH 7.5, 3.8 mM $MgCl_2$, 2 mM ATP, 0.5 mM GTP, 0.2 mg/mL creatine kinase, 12 mM creatine phosphate, 165 mM KAc, 100 μM amino acid minus methionine, 30 μM methionine and 0.17 μM [^{35}S]-methionine (1Ci/μmol) (9). 50 nM of α-sarcin and 12.5 μL of WT or ΔP1P2 S30 (same absorbance at 260 nm) were added. Translation reactions were performed at 25ºC for 30 min and stopped and analyzed as described above.

Heterologous expression of α-sarcin in *S. cerevisiae* and toxicity assays. The effects of wild type and mutant α-sarcin cytoplasmic expression in wild-type, ΔP1αP2β and ΔP2 *S. cerevisiae* strains were analyzed in solid and liquid media. Viability of several fresh transformants was assayed in SC-agar medium lacking tryptophan (SC-W) with and without inducer (5 μg/mL doxycycline) by spotting 4 μL aliquots containing 10^4 cells and 10-fold serial dilutions (11). For liquid growth experiments, overnight SC-W cultures of the indicated strains were checked for cell viability (by CFU culture on SC-W plates) and incubated at an initial concentration of 10^7 cells/mL and successive 2-fold serial cell dilutions on SC-W 96-wells plates (with or without 30 μg/mL doxycycline) for 18 h (WT), 24 h (ΔP1αP2β) or 28 h (ΔP2) at 30ºC with shaking at 100 rpm. The OD_{600} reached was taken as an indicative of growth. The presence and ribonucleolytic activity of ribotoxins were analyzed from liquid cultures. Yeast strains were allowed to grow up to 0.1 OD_{600}, and then doxycycline induced for 4-6 hours. Finally cells were harvested and RNA and

DNA extracted for poison primer extension and α-sarcin specific PCR analysis respectively.

Statistical analysis. All *in vitro* results correspond to at least triplicate experiments. *In vivo* liquid growth was repeated at least two times. Statistical analyses were carried out by using the statistics utility of the Sigma Plot v11.2 Program (Systat Software, Erkrath, Germany). ANOVA analyses were used for comparison of multiple groups of factors and t-tests for two sets of data. All In all cases Shapiro-Wilk normality tests were passed. Statistical significance was considered to be achieved at the $p<0.05$ level.

SUPPLEMENTARY FIGURES

Figure S1.- Schematic representation of the ribosomal stalk composition from bacteria and yeast for comparison. Main differences appear at the flexible C-termini domains of L10 versus P0 and L7/12 versus P1 and P2.The different wild-type and mutant *S. cerevisiae* ribosomes used along this study are also included. Names of yeast strains are within brackets. D45 strain has P2 proteins deleted from its genome destabilizing both P1 and showing the same phenotype as D4567.

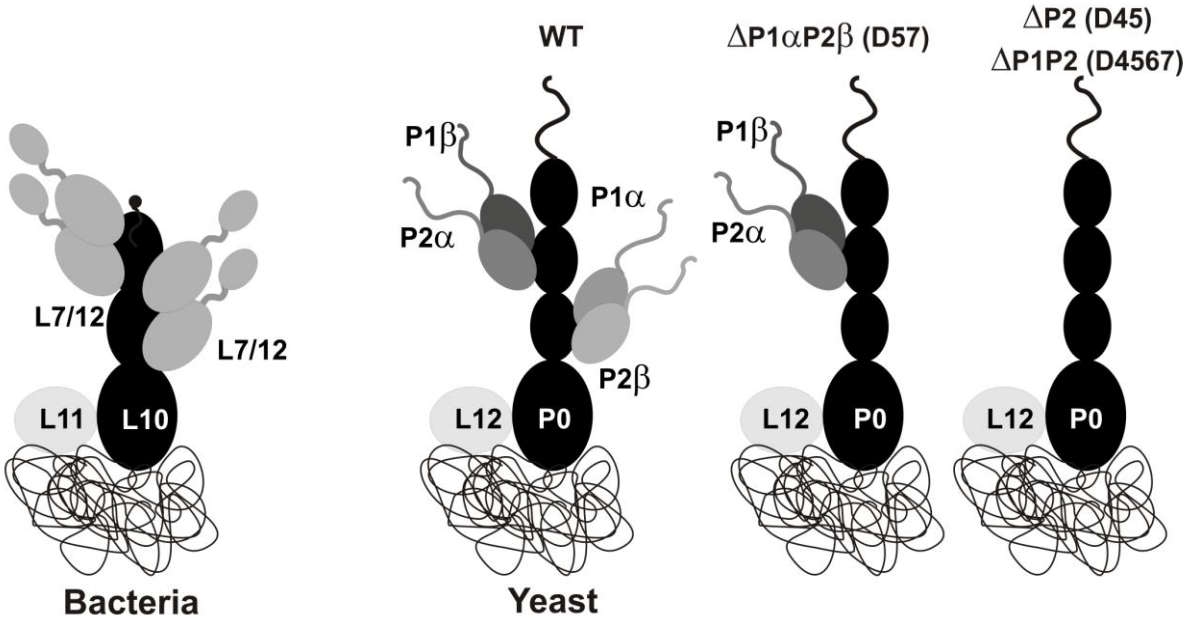


Figure S2.- Effect of the expression of wild-type α-sarcin and its two non-toxic mutants in WT *S. cerevisiae*. (–) W303-pCM175; (sarcin) W303-pCM175αS; (sarΔ) W303-pCM175αSΔ(7-22); and (sar3M) W303-pCM175αS H50/137Q/E96Q strains were grown in SC-W without (left panel) and with (right panel) 5 μg/mL doxycycline (DOX). PhosphorImager result of poison primer extension of extracted RNA. Bands due to intact 25S, α-sarcin specific cleavage (α) and excess of primer are indicated.

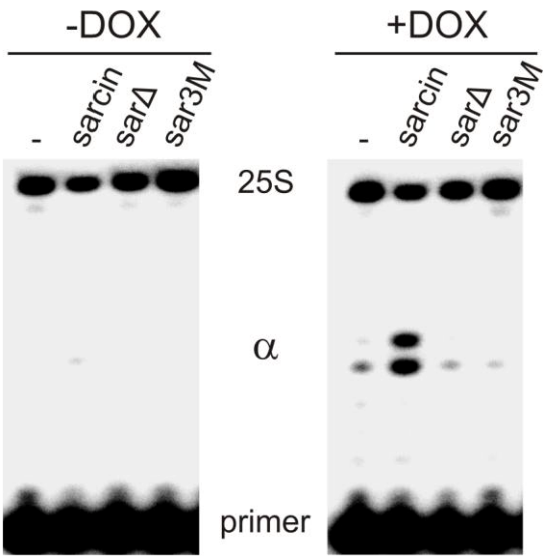
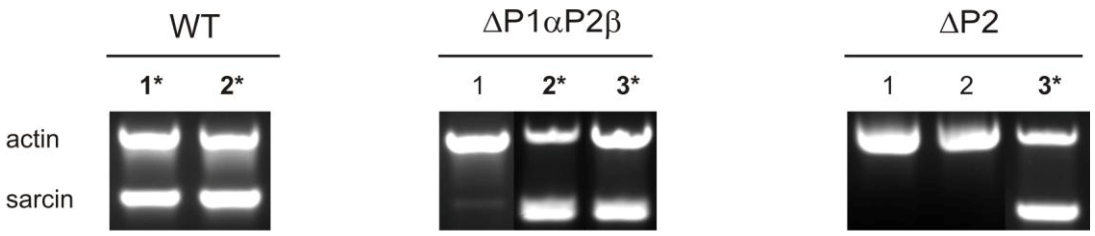


Figure S3.- α-Sarcin cDNA PCR amplification of clones shown in Fig. 2C. A positive control of actin DNA level was included.



ADDITIONAL REFERENCES

1. Remacha, M., Santos, C., Bermejo, B., Naranda, T. and Ballesta, J.P. *J Biol Chem.* **1992**, *267*, 12061-12067.
2. Remacha, M., Jiménez-Díaz, A., Bermejo, B., Rodriguez Gabriel, M.A., Guarinos, E., and Ballesta, J.P. *Mol. Cell. Biol.* **1995**, *15*, 4754-4762.
3. Lacadena, J., Martínez del Pozo, A., Martínez-Ruiz, A., Pérez-Cañadillas, J.M., Bruix, M., Mancheño, J.M., Oñaderra, M., and Gavilanes, J.G. *Proteins* **1999**, *37*, 474-484.
4. García-Ortega, L., Masip, M., Mancheño, J.M., Oñaderra, M., Lizarbe, M.A., García-Mayoral, M.F., Bruix, M., Martínez del Pozo, A., and Gavilanes, J.G. *J. Biol. Chem.* **2002**, *277*, 18632-18639.
5. Garí, E., Piedrafita, L., Aldea, M., and Herrero, E. *Yeast* **1997**, *13*, 837-848.
6. Bellí, G., Garí, E., Piedrafita, L., Aldea, M., and Herrero, E. *Nucleic Acids Res.* **1998**, *26*, 942-947.
7. Lacadena, J., Martínez-del-Pozo, A., Barbero, J.L., Mancheño, J.M., Gasset, M., Oñaderra, M., López-Otín, C., Ortega, S., García, J., and Gavilanes, J.G. *Gene.* **1994**, *142*, 147–151.
8. Rodríguez-Gabriel, M.A., Remacha, M., and Ballesta, J.P. *Biochemistry.* **1998**, *37*, 16620-16626.
9. Henderson, A., and Hershey, J.W. *Proc. Natl. Acad. Sci. U S A.* **2011**, *108*, 6415-6419.
10. García-Ortega, L., Álvarez-García, E., Gavilanes, J.G., Martínez-del-Pozo, A., and Joseph, S. *Nucleic Acids Res.* **2010**, *38*, 4108-4119.
11. Chiou, J.C., Li, X.P., Remacha, M., Ballesta, J.P., and Tumer, N.E. *Mol. Microbiol.* **2008**, *70*, 1441-1452.

C2. Las partículas pre-60S en estados avanzados de maduración pueden ser inactivadas por la α-sarcina.

La biogénesis del ribosoma eucariota es un proceso complejo que conlleva el ensamblaje, maduración y transporte intracelular de las subunidades del ribosoma. Las partículas pre-60S y pre-40S viajan por el nucleoplasma hacia el complejo del poro nuclear y una vez en el citosol se suceden las etapas finales de maduración. Las ribotoxinas fúngicas actúan sobre el SRL, una secuencia de rRNA conservada de la subunidad 60S que juega un papel clave en la función del ribosoma. El corte del SRL por parte de estas toxinas impide que el ribosoma interaccione con los factores de elongación necesarios para que se produzca la síntesis de proteínas.

En este trabajo se analiza a nivel molecular el efecto de la actividad de la ribotoxina α-sarcina sobre la ruta de maduración del ribosoma eucariota, utilizando la levadura *Saccharomyces cerevisiae* como organismo modelo. El trabajo realizado ha permitido concluir que, a pesar de que la a-sarcina puede acceder al núcleo y, por tanto, a cualquier partícula prerribosomal, el SRL sólo es susceptible a la acción de ésta cuando forma parte del rRNA 25S ya maduro, en las partículas pre-60S en los últimos estadíos de la biogénesis. A largo plazo, el uso de las ribotoxinas en este campo podría contribuir a un mejor conocimiento de las enfermedades relacionadas con defectos en el ribosoma, las denominadas ribosomopatías.

Trabajo C2: Olombrada M, Altvater M, Peña C, Nerurkar P, Martínez del Pozo A, Gavilanes JG, García Ortega L y Panse VG (2015). "Fungal ribotoxins as tools for the study of ribosome biogenesis in yeast" En preparación.

Late pre-60S ribosomes can be inactivated by α-sarcin

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Abstract

Eukaryotic ribosomes are assembled in an extraordinarily complex pathway that requires coordination of multiple factors. While travelling from the nucleoplasm across the nuclear pore complex to finally reach the cytoplasm, ribosomal particles undergo several changes in composition, both at the protein and rRNA level. During cytoplasmic maturation, pre-60S particles already have mature 25S rRNA, which makes them susceptible of being inactivated by ribotoxins. This family of RNases inhibit protein synthesis by cleaving the sarcin/ricin loop (SRL), a conserved rRNA structure essential for translation. Here we show for the first time how the ribotoxin α-sarcin can inactivate not only mature ribosomes, but also pre-60S particles during cytoplasmic maturation. Both *in vivo* and *in vitro* assays revealed how rRNA composition of pre-60S particles is important for the activity of α-sarcin. We have also studied the effect of SRL cleavage on the nuclear export and recycling of ribosomal subunits and assembly factors. Together, our data suggest that α-sarcin is able to attack late pre-60S particles without disturbing the general maturation pathway, evidencing that the proper folding of the SRL might be assessed later on the maturation process.

Keywords: Ribosome biogenesis, *Saccharomyces cerevisiae*, α-sarcin, rRNA

Abbreviations: SRL, sarcin-ricin loop; RNase, ribonuclease; RNP, ribonucleoprotein; RP, ribosomal protein; BME, β-mercaptoethanol; TAP, Tandem Affinity Purification; ITS, Internal Transcribed Spacer; PAP, Pokeweed antiviral protein

Introduction

The ribosome is an essential ribonucleoprotein (RNP) which acts as a ribozyme involved in the translation of the genetic information into proteins. All ribosomes are comprised of two subunits with a different composition of rRNAs and ribosomal proteins (RPs). In yeast, the small 40S subunit contains one molecule of RNA (18S rRNA) and 33 different RPs, and is responsible of decoding the genetic information by bringing together mRNA and tRNAs, whereas the large 60S subunit, where the peptidyltransferase reaction occurs, is composed of three rRNAs (5S, 5.8S and 25S) and

46 RPs. The structure of the mature yeast ribosome is known at the molecular level (Ben-Shem et al. 2011), however, the process by which this cellular machine is assembled is not completely well understood.

Ribosome biogenesis is a complex and highly regulated process. Eukaryotic ribosomes are initially assembled in the nucleolus, where the rRNA is transcribed. Then, further maturation takes place also in the nucleoplasm and cytoplasm. In *Saccharomyces cerevisiae*, more than 300 trans-acting factors, both proteins and RNAs, participate in the assembly, maturation and intracellular transport of ribosomal subunits. In humans this number is even higher, according to the bigger size and complexity of their ribosomes (Gerhardy et al. 2014, Lafontaine 2015, Woolford and Baserga 2013). Assembly begins with transcription of 35S pre-rRNA and 5S RNA by RNA polymerases I and III, respectively. Post-transcriptional modifications of the 35S pre-rRNA, as well as association with RPs and trans-acting factors, give rise to the earliest ribosomal precursor, the 90S pre-ribosome. This particle is then cleaved, releasing pre-40S and pre-60S particles that each undergo independent maturation pathways. Pre-40S particles are transported across the nuclear pore complex going through only a few compositional changes in the nucleoplasm, and it is in the cytoplasm where final maturation steps happen, including processing of the 20S pre-rRNA to mature 18S rRNA. Maturation of the pre-60S particle involves more processing steps within the nucleoplasm, so that once it is exported to the cytoplasm only a few factors remain attached to the particle. These factors are sequentially released from the pre-60S particles, including major steps like the release of factors from the exit tunnel, the assembly of the stalk structure (RPs P0, P1 and P2) and the release of the anti-association factor Tif6 (de la Cruz et al. 2015, Panse and Johnson 2010, Woolford and Baserga 2013). The rRNA molecules that form part of the 60S subunit are processed mainly in the nucleoplasm. Cleavage of the 35S pre-rRNA precursor generates a 27S pre-rRNA that is further processed to 25S rRNA and 7S pre-rRNA in the nucleoplasm through several alternative pathways, being the latter one finally processed to mature 5.8S in the cytoplasm (Gerhardy et al. 2014, Woolford and Baserga 2013). Generation of new ribosomes is generally linked to cell proliferation. For that reason, misregulation of this complex chain of events involved in ribosome biogenesis can be lethal and can lead to a variety of human diseases known as ribosomopathies (Freed et al. 2010, Narla and Ebert 2010).

Ribotoxins are a family of toxic extracellular fungal RNases that exert a highly specific ribonucleolytic activity on the larger molecule of rRNA in the ribosome, leading to protein synthesis inhibition and cell death (Lacadena et al. 2007, Olmo et al. 2001). They were discovered in the 1960's during a screening program searching for antibiotics and antitumor agents (Olson and Goerner 1965). Nowadays, some of these toxins have been thoroughly studied and characterized: α-sarcin, the first to be discovered, restrictocin, Aspf1 and hirsutellin A (HtA), the smallest ribotoxin known (Herrero-Galán et al. 2008, Lacadena et al. 2007). Unfortunately, although discovered

as antitumor molecules, further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses and prompted the abandon of their study until the mid-1970's. Later on, it was observed that they inhibited protein biosynthesis in ribosomal preparations by cleaving only one phosphodiester bond of the large rRNA molecule (the bond between G4325 and A4326 of the 28S rat ribosome, G2661-A2662 in the *Escherichia coli* ribosome), (Schindler and Davies 1977, Endo and Wool 1982). This bond is of particular interest, because it is located at a universally conserved site, the sarcin-ricin loop (SRL), with important roles in ribosome function (García-Ortega et al. 2010). The toxic effect of ribotoxins has been related to the inability of the cleaved ribosomes to interact in optimum conditions with the elongation factors needed for a correct and efficient protein biosynthesis (García-Ortega et al. 2010). The activity of ribotoxins has been investigated mainly for their ability to cleave mature ribosomes of different origins (Olombrada et al. 2014a, Olombrada et al. 2014b). The ability of these toxins to act on immature pre-60S particles is an interesting issue that has not been studied until now. The present work addresses the activity of fungal ribotoxins at different steps of the maturation pathway, showing for the first time how these toxins can also inactivate immature ribosomes.

Materials and methods

Bacteria and yeast strains, plasmids and ribotoxins

All recombinant DNA techniques were performed according to established procedures using *Escherichia coli* DH5αF' and XL1-Blue strains for cloning and plasmid propagation. Yeast strains and plasmids used in this study are listed in Supplementary Tables 1 and 2, respectively. All cloned DNA fragments generated by PCR amplification and plasmids were verified by sequencing at the corresponding Complutense University facility. Wild type α-sarcin and several mutant variants were cloned under the control of the galactose promotor: a deletion version, α-sarcin Δ(7-22), which is active but unable to specifically cleave the SRL, and four catalytically inactive mutants, H50Q, H50/137Q, H50/137Q/E96Q and R121Q (Lacadena et al. 1999, Masip et al. 2001). Wild type fungal α-sarcin was produced from the mold *Aspergillus giganteus* and purified as previously described (Lacadena et al. 1994).

Cell lysates and northern blot

The activity of α-sarcin was assayed on wild type yeast cell lysates. Briefly, yeast cells were grown in 200 ml of YPD media and harvested when reach OD_{600} 0.75. Then, cells were washed twice with lysis buffer (30 mM Hepes-NaOH pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$) and resuspended in 1 ml of lysis buffer. Glass beads were added and cells lysed by vortexing at room temperature and maximum speed for 6 min. To recover the lysate, a hole was made through at the bottom of the tube and the lysate

was collected in a new tube by pushing the lysate using a syringe plug, so the glass beads remain in the old tube. The collected lysate was clarified by centrifugation at 14000 rpm and 4ºC for 10 min and concentration of RNA was measured. Reactions were performed in 25 μL of 30 mM Hepes-NaOH pH 7.5, 30 mM KCl, 20 mM NaCl, 8 mM $MgCl_2$ and 5 mM BME during 13 min, containing 10 μl of lysate (15 μg of RNA approximately) and different concentrations of α-sarcin (0-160 nM). Afterwards, α-sarcin reactions were stopped by adding Proteinase K to the mix for 15 min at 42ºC followed by RNA extraction with phenol-chloroform-isoamyl alcohol and precipitation in isopropanol. RNA pellets were washed with 80 % ethanol and resuspended in 15 μl water. 1 μg of total RNA was separated on a 1.2 % agarose/formaldehyde gel for 1.5 h at 200 V. RNA was transferred to a Nylon membrane (Hybond-N^+; GE Healthcare) by capillary blotting and subsequently UV cross-linked to the membrane (Stratalinker 1800; Agilent Technologies). The membrane was then subjected to Northern Blot analysis using a 5'-radioactively-labeled probes complementary to the sequence of the 25S rRNA (5'-ACAAATCAGACAACAAAGGCTTAA-3') and the 27S pre-rRNA (5'-GAAAAGGCCAGCAATTTCAAGTTA-3'). Results were visualized using a PhosphorImager screen.

Heterologous expression of α–sarcin in S. cerevisiae

The effects of cytoplasmic expression of wild type and mutants of α-sarcin was analyzed in solid and liquid media. Viability of several fresh transformants was assayed by spotting 10-fold dilutions on glucose (SD-URA), raffinose (SR-URA) or galactose (SG-URA) plates at 20ºC, 30ºC and 37ºC for 3-7 days. For detection of the ribonucleolytic activity of ribotoxins in liquid media, yeast were allowed to grow in the presence of glucose until they reached OD_{600} 0.7-0.9, and ribotoxin expression was induced by adding 20% galactose to the culture. After different times of induction, RNA was extracted with phenol-chloroform-isoamylalcohol and results were analyzed by northern blot using a radioactive-labeled probe complementary to the 25S rRNA.

Tandem Affinity Purification

Tandem Affinity Purification (TAP) of pre-ribosomal particles were carried out as previously described (Altvater et al. 2014) with slight modifications. Briefly, after incubation with TEV, the eluate is split into two, adding 40 nM final concentration of α-sarcin to one of them. The eluates are then incubated at 30ºC for 30 min, and then continued TAP protocol as previously described by Altvater et al. 2014. Calmodulin-eluates were separated on NuPAGE 4-12 % Bis-Tris gradient gels (Invitrogen, Carlsbad, CA, USA) and visualized by silver staining. To analyze RNAs after TAP purification, RNA was extracted with phenol-chloroform-isoamyl alcohol after α-sarcin's treatment and from calmodulin-eluates and precipitated in isopropanol. RNA was then analyzed by

northern blotting with radioactive-labeled probes complementary to the 25S and 27S rRNA sequence.

Fluorescence in situ hybridization (FISH) and microscopy

uL18-GFP (RPL5-GFP) and uS5-GFP (RPS2-GFP) reporter assays were performed to analyze pre-ribosomal subunit export as described in Altvater et al. 2014. Localization of 20S pre-rRNA was analyzed by FISH using a Cy3-labeled oligonucleotide probe (5'-Cy3-ATG CTC TTG CCA AAA CAA AAA AAT CCA TTT TCA AAA TTA TTA AAT TTC TT-3') that is complementary to the 5' portion of ITS1 as described (Altvater et al. 2014).

Cells were visualized using a DM6000B microscope (Leica, Germany) equipped with HCX PL Fluotar 63x/1.25 NA oil immersion objective (Leica, Solms, Germany). Images were aquired with a fitted digital camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, SZK, Japan) and Openlab software (Perkin-Elmer, Waltham, MA, USA).

Results

α–sarcin does not cleave 27S pre-rRNA in vitro.

The activity of the ribotoxin α-sarcin was assayed using as substrate a cell extract from a wild type strain of *Saccharomyces cerevisiae*. Analysis of the rRNA of the samples by northern blotting indicated that, as already established, mature 25S rRNA was specifically cleaved by the toxin. The rRNA fragment produced by the unique and highly specific activity of ribotoxins, known as the α-fragment, was detected in a dose-dependent manner, and at all of the concentrations of α–sarcin tested, using a specific probe for the 25S rRNA (Figure 1). On the other hand, this α-fragment was not detected when the probe was specific for the 27S pre-rRNA (Figure 1), and the 27S pre-rRNA band remained intact at any of the concentrations assayed, indicating that it was not being cleaved by the toxin. To further investigate this result, a GFP-α–sarcin version was expressed in a wild type yeast strain and its intracellular location was analyzed, showing that the expressed toxin localized both to the cytoplasm and the nucleus (data not shown). Thus, it seemed that the toxin can move freely from the cytoplasm to the nucleus indicating that the lack of 27S pre-rRNA cleavage was not due to a lack of accessibility. Therefore, this suggested that either the composition of the pre-60S particles or the processing of pre-rRNA had to be critical for SRL availability to the toxin. With this idea in mind, four different pre-60S particles, characteristic of different steps of ribosome maturation, were purified using Tandem Affinity Purification (TAP), adding an additional incubation step with α-sarcin during the purification. Ssf1-TAP purifies early nucleolar pre-60S particles, Rix1-TAP and Arx1-TAP are mainly intermediate nucleoplasmic and late pre-60S subunits, and Kre35-TAP refers to cytoplasmic pre-60S particles. Protein composition, as well as rRNA integrity, were compared in these samples, treated or not with the toxin (Figure 1B). At the protein level, no substantial differences were observed between pre-60S particles.

However, RNA extraction and northern blot revealed how the α-fragment was preferentially released at the late maturation steps of pre-60S particles (Arx1-TAP and Kre35-TAP), where rRNA has been already processed to mature 25S. In early pre-60S particles (Ssf1-TAP and Rix1-TAP) the α-fragment was only scarcely released and given that 27S pre-rRNA is much more abundant in these particles, it is then safe to interpret that α-sarcin is not cleaving this pre-rRNA as effectively as it does for late pre-60S.

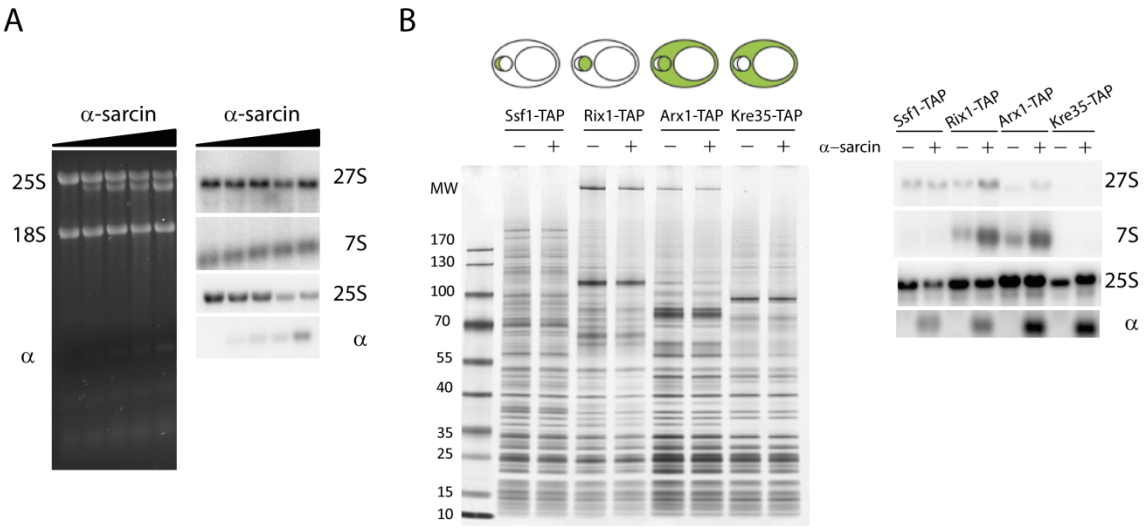


Figure 1.-Ribotoxins differentially cleave mature and pre-rRNAs. A) α-Sarcin activity on yeast cell lysates. Total RNA was extracted and analyzed by agarose gel (leftpannel) and northern blot (right pannel) after incubation of the lysate for 13 min with increasing amounts of α-sarcin (0-160 nM). Specific probes for the 25S rRNA and 27S pre-rRNA were used to detect the cleavage of SRL by northern blot. The probe for 25S was also used to detect the characteristic fragment released after SRL cleavage (α, for α-fragment). B) Analysis of the activity of α-sarcin on pre-60S particles. TAP purified pre-60S particles incubated or not with 40 nM of α-sarcin during the purification were separated on 4-12 % gradient gels and subjected to silver staining, and C) to northern analysis using the same probes as in (A) for the 25S rRNA and 27S pre-rRNA.

In vivo expression of α-sarcin leads to rRNA cleavage

Heterologous expression in the yeast *S. cerevisiae* of wild type α-sarcin and five mutant variants affecting either the catalytic activity (mutants H50Q, H50/137Q, H50/137Q/E96Q and R121Q) or the specific recognition of the ribosome (mutant Δ[7-22]) was then analyzed. All these protein mutants have been thoroughly characterized (García-Mayoral et al. 2004, García-Ortega et al. 2002, Lacadena et al. 1999, Masip et al. 2001) showing that they retain the overall structural features of the wild-type protein. Their expression was under the control of the GAL promoter, which is activated exclusively in the presence of galactose. In solid media, yeast growth was inhibited at all tested temperatures when wild type α-sarcin was expressed, whereas expression of the mutants, in general, did not affect growth (Figure 2A), as previously described in a similar system (Olombrada et al. 2014b). Interestingly, production of Δ(7-22) at 20ºC inhibited yeast growth approximately as much as the wild type toxin.

In fact, close inspection of these results reveals that wild-type α-sarcin is equally effective at the three temperature values assayed, revealing that the amount of toxin produced is far above the levels needed to completely inhibit yeast growth in the conditions used. Given that the Δ(7-22) mutant retains its non-specific catalytic activity (García-Ortega et al. 2002) it seems clear that the pool of active ribosomes is more easily affected by the toxin at the lowest temperature assayed. Growth inhibition was correlated with detection of α-sarcin's specific catalytic activity, as revealed by the detection of the characteristic α-fragment in liquid cultures, even at short times of induction (Figure 2B).

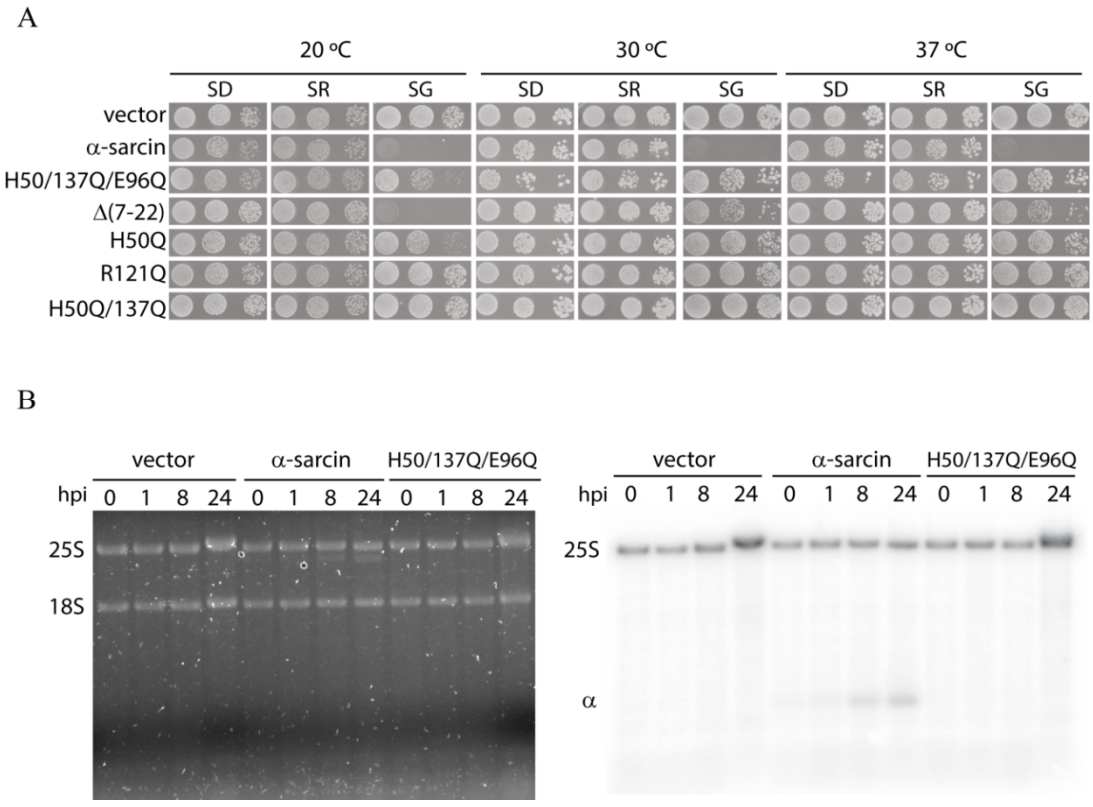


Figure 2.-Heterologous expression of ribotoxins in yeast. A) Wild type yeast cells transformed with plasmids containing α-sarcin or different mutant variants were spotted in 10-fold serial dilutions on glucose (SD), raffinose (SR) or galactose (SG) plates and grown at indicated temperatures for 3-6 days. Growth of yeast was inhibited when the expression of wild type α-sarcin was induced. (B) Growth inhibition is correlated to the activity of ribotoxins. Total RNA of liquid cultures expressing ribotoxins was analyzed by agarose electrophoresis (left pannel) and northern blot (right pannel) using a specific probe for the 25S rRNA. The characteristic α-fragment released by ribotoxins was detected by northern blot only when wild type α-sarcin was expressed.

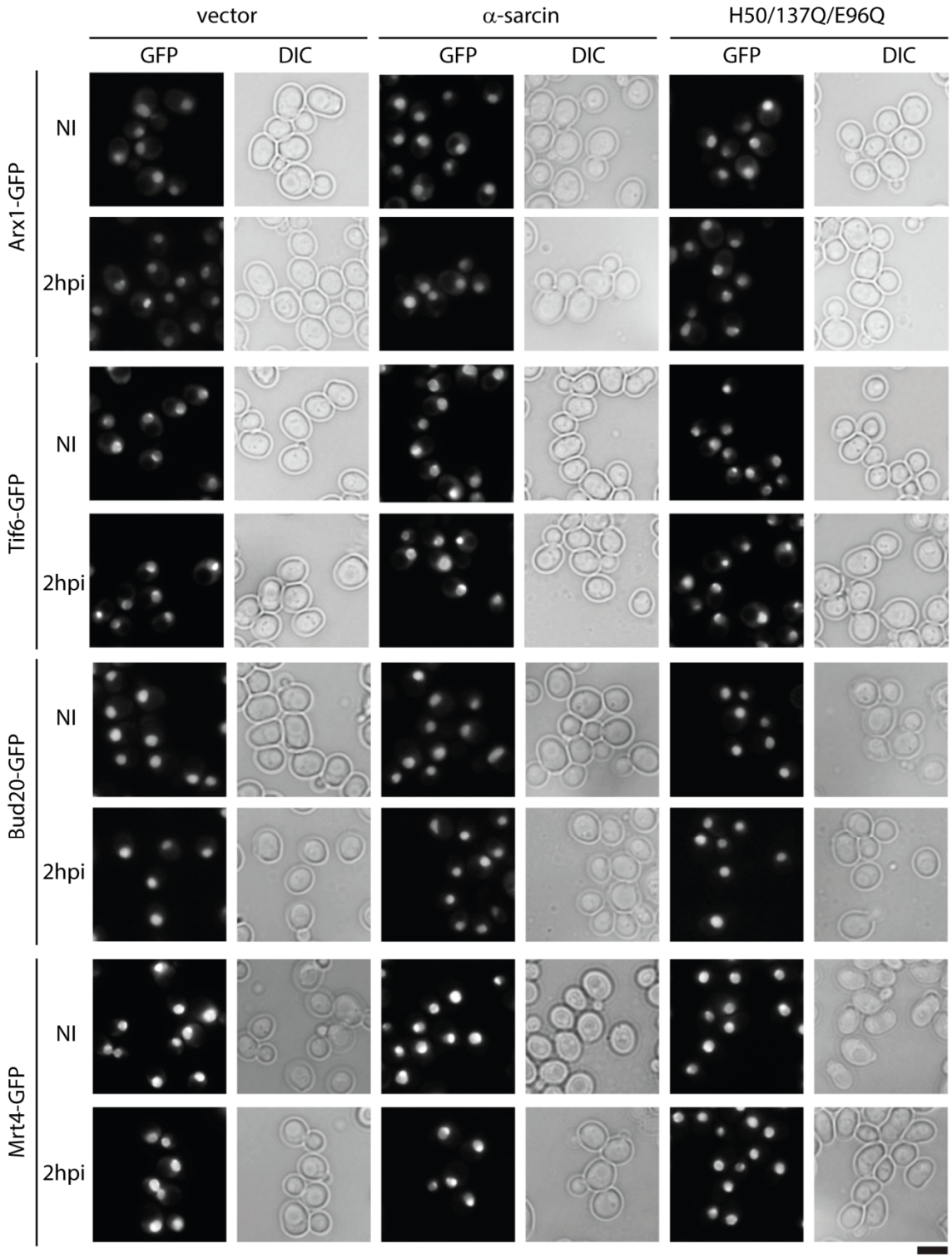


Figure 3.- Recycling of trans-acting factors is not affected by α-sarcin expression. Yeast strains containing different GFP-tagged trans-acting factors (Arx1, Bud20, Tif6, Mrt4) were transformed with plasmids containing α-sarcin or the catalytically inactive mutant H50/137Q/E96Q. Intracellular location of these factors was analyzed by fluorescence microscopy before (NI) and after 2 hours of induction (2hpi) of ribotoxin expression. Empty vector was used as control. Scale bar=5 μm.

Intracellular expression of α-sarcin does not affect recycling of trans-acting factors

Yeast strains producing different GFP-tagged trans-acting factors were transformed with plasmids with the ability to produce either wild type α-sarcin or its catalytically inactive mutant H50/137Q/E96Q. Wild type α-sarcin was always able to inhibit yeast growth and produce the α-fragment, as shown before for the wild type yeast strain (Supplementary figure 1). However, in this case, and taking advantage of the presence of the GFP tag as fusion partner, it was also possible to study the intracellular localization of these factors. Thus, the microscopy-based analysis of these strains, before and after 2 hours post-induction of ribotoxin's expression, revealed no significant mislocalization of GFP-fluorescence (Figure 3, Supplementary figure 2). Therefore, it could be safely assumed that recycling of these factors during ribosome maturation was not being affected by α-sarcin toxicity.

Ribotoxin expression induces accumulation of 35S pre-rRNA in the nucleolus

To check whether the activity of ribotoxins was somehow affecting processing of the small subunit 20S pre-rRNA, we performed fluorescence in situ hybridization (FISH) using a specific probe for the ITS1 rRNA sequence. This possibility was discarded given that, upon ribotoxin induction, ITS1-flourescence signal remained nucleolar (Figure 4). Interestingly, the size of the nucleolus seemed larger in those cells expressing both ribotoxin versions, wild-type and H50/137Q/E96Q, when compared to the control (Figure 4). This result suggested enhancement of 35S pre-rRNA transcription, probably as a compensatory mechanism due to the reduction in the number of functional 60S subunits in the cytoplasm.

The activity of ribotoxins does not affect nuclear export of 60S and 40S pre-ribosomes

Finally, to investigate whether SRL cleavage by ribotoxins was affecting export of ribosomal subunits, the localization of known 40S (uS5-GFP) and 60S (uL18-GFP) reporters in a wild type strain were examined. As expected, wild type cells showed cytoplasmic localization for both of them (Figure 5) and this localization was not affected upon induction of ribotoxin expression (Figure 5), in clear contrast with the effect observed in the control strains (bud20Δ accumulates uL18-GFP and yrb2Δ accumulates uS5-GFP in the nucleus). Overall, these results suggest that the integrity of the SRL is not essential for nuclear export of both pre40S and pre-60S subunits

Discussion

α-sarcin specific cleavage takes place only at the late stages of ribosomal maturation

For decades, the study of fungal ribotoxins has been focused only on the activity they display on mature prokaryotic and/or eukaryotic ribosomes. However,

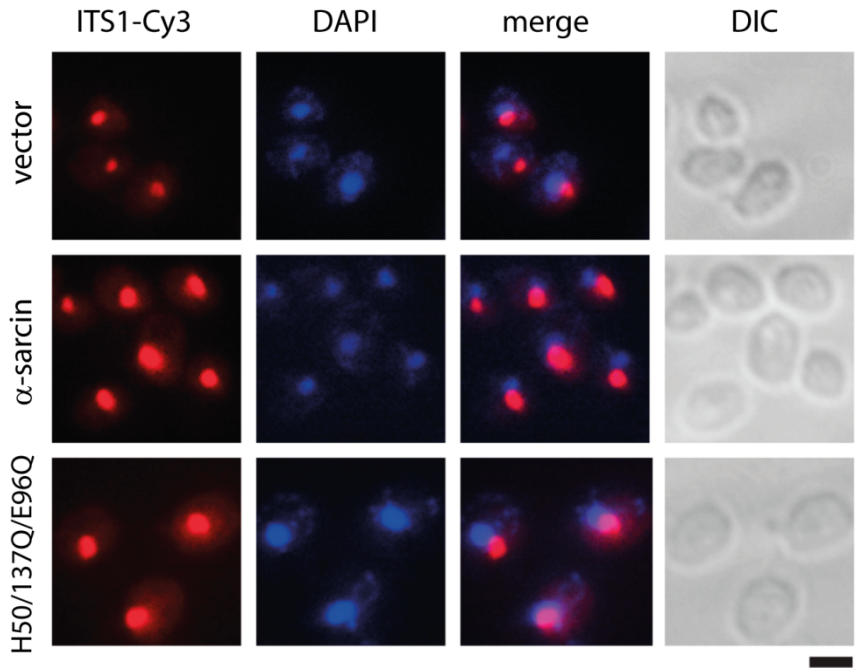


Figure 4.-Fluorescence *in situ* hybridization (FISH) was performed using a Cy3-labelled oligonucleotide complementary to the 5' portion of ITS1 rRNAsequence (red). Nuclear DNA was stained with DAPI (blue). Wild type yeast cells show no mislocalization of the ITS1 signal after 3.5 hours post-induction of α-sarcin or the H50/137Q/E96Q mutant. Cells were grown at 30ºC to mid-log phase after addition of galactose. Empty vector was used as a control. Scale bar =5 μm.

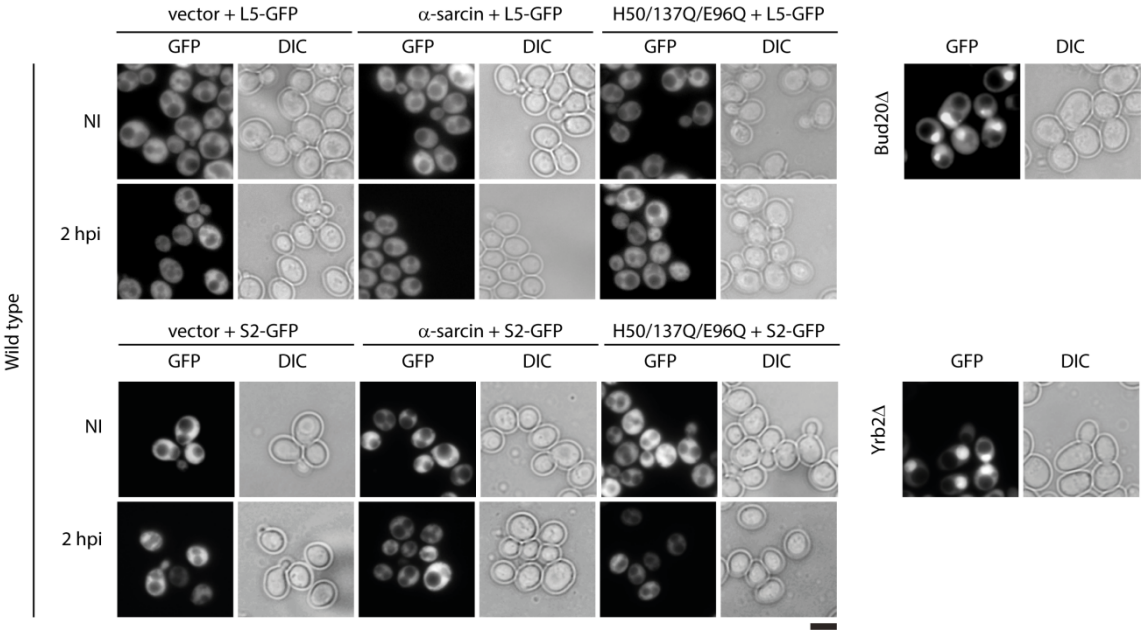


Figure 5.- Wild type cells expressing the indicated GFP fusion proteins and α–sarcin or the inactive mutant H50/137Q/E96Q were grown at 30ºC until mid-log phase. Cells were analyzed by fluorescence microscopy before (NI) and after 2 hours post ribotoxin induction (2hpi). The yrb2Δ and bud20Δ mutants were used as controls for uS5-GFP and uL18-GFP mislocalization, respectively. Scale bar=5μm.

analysis of their potential effect on eukaryotic ribosome maturation is of much interest as it can contribute not only to our understanding of their mechanism of action but also to the elucidation of this maturation pathway. The ubiquitous intracellular localization of α-sarcin observed when expressed in yeast is in agreement with previous results published by Alford et al. 2009 that showed how this protein can enter the nucleus of mammalian cells. This ability would allow α-sarcin to contact early pre-60S particles, making them potential targets for this toxin. However, it is now demonstrated how the nuclear localized 27S pre-rRNA cannot be cleaved by this toxin (Figure 1A). This observation could be explained by two different non-exclusive reasons. First, the SRL might not be yet sufficiently structured at these early stages of maturation as to be specifically recognized by α-sarcin. Second, perhaps the SRL is structured but somehow protected by one or several of the trans-acting factors that drive ribosome maturation in the nucleus, or maybe by other rRNA elements. Both options would explain why SRL cleavage is hindered.

Indeed, the behavior of α-sarcin is quite different depending on the nature of the various TAP-purified pre-60S particles. Thus, the release of the characteristic α-fragment of SRL is more evident in late pre-60S particles like those associated to Kre35-TAP or Arx1-TAP than in early ones such as those represented by SSf1-TAP or Rix1-TAP (Figure 1B). These early particles are enriched in 27S pre-rRNA, which then would remain intact, confirming the results shown above. Overall, it seems safe to conclude that fungal ribotoxins have preference for mature 60S or late pre-60S ribosomal particles, being catalytically incompetent against 27S pre-rRNA.

In vivo expression of α-sarcin in yeast leads to rRNA cleavage but does not affect recycling of trans-acting factors

In order to validate the results obtained in vitro with the TAP particles and cell lysates, a yeast in vivo model was tested. In such model, the ribotoxin α-sarcin and several mutant variants were expressed under the control of a galactose inducible promoter. Production of wild type α-sarcin resulted in growth inhibition at any of the three temperatures checked (Figure 2A), a behavior that was not observed for the catalytically inactive mutants. This growth inhibition was indeed correlated with the release of the α-fragment (Figure 2B), as published before using a different inducible system (Olombrada et al. 2014b). In good agreement with all these results, this α-fragment was not detected in cells expressing the empty vector or the catalytically inactive mutant H50/137Q/E96Q, where no growth defect was found.

Interestingly, the deletion mutant Δ(7-22) also inhibited growth at low temperatures. This mutant is defective in specifically recognizing the ribosome but still retains ribonucleolytic non-specific activity, what could explain the observed cytotoxicity at low temperatures. Once the in vivo system was validated, the main goal was to check whether some events related to ribosome biogenesis could be affected

by the expression of the toxin. Starting with recycling of export and assembly factors, the yeast strains containing different GFP-tagged trans-acting factors did not show any significant differences in terms of localization (Figure 3 and Supplementary figure 2). It should be concluded then that this particular event is not affected by the activity of α-sarcin. In fact, there was inhibition of growth in all the strains expressing wild type α-sarcin (Supplementary figure 1), and the α-fragment could be detected in all of them as early as 2 hours after induction (data not shown), proving that SRL cleavage does not affect trans-acting factors localization. Not even that of Tif6, which has been described to be located in the vicinity of the SRL and interacts with RP uL14 (Greber et al. 2012, Klinge et al. 2011), a protein that is predicted to be recognized by α-sarcin (García-Mayoral et al. 2005). Most of these factors, if not all, were present in the TAP-purified particles, especially in Arx1-TAP and Kre35-TAP. Since α-sarcin was able to cleave the 25S rRNA in all of them, it should be concluded that the presence of factors like Tif6 and Nmd3, in close proximity to the SRL, do not preclude SRL accessibility by the toxin.

Intracellular production of α-sarcin does not cause pre-20S rRNA processing defects but stimulates 35S pre-rRNA accumulation in the nucleolus

The target of ribotoxins is the SRL, a highly conserved motif located in the 60S ribosomal subunit. Consequently, cleavage of this rRNA structure could trigger defects at different stages of the maturation pathway. For example, lack of translational competent 60S subunits in the cytoplasm, due to inactivation by ribotoxins, could induce a compensatory effect affecting maturation of 40S subunits. Within this idea, we tested the possibility of SRL cleavage affecting 20S pre-rRNA processing to mature 18S rRNA. FISH experiments showed, however, that this maturation event was not altered upon ribotoxins production (Figure 4). However, these cells showed a stronger signal in the nucleolus. An explanation for this observation could be the induction of massive transcription of 35S pre-rRNA, most likely as a direct consequence of SRL cleavage. The activity of α-sarcin on 60S subunits in the cytoplasm would reduce the amount of 60S able to enter translation, and the nucleus would start transcribing 35S pre-rRNA in order to compensate this effect.

On the other hand, microscopy analysis of the 40S reporter uS5-GFP revealed no accumulation of 40S subunits in the nucleus after α-sarcin induction (Figure 5), suggesting that the activity of the ribotoxin did not affect the maturation pathway of these particles. Accordingly, no nuclear accumulation was observed for the 60S reporter uL18-GFP upon induction of α–sarcin (Figure B). This implies that the integrity of the SRL is not required for the correct export of pre-60S subunits, and cleaved pre-60S particles could be exported to the nucleus without problems even though they will not be functional. In general, it seems that SRL cleavage by ribotoxins does not affect ribosome maturation in the nucleus, and major effects would most likely occur in the cytoplasm.

The correct assembly of the ribosome is extremely important to ensure the appropriate function and fidelity of translation, that is why cells must have designed strategies to assess this assembly. The release of Tif6 by Efl1 from the nascent 60S subunit has been proposed as one of these check points (Bussiere et al., 2012). Efl1 would be recruited to the pre-60S particle once the stalk is assembled and there it would check the integrity of the P-site. This would then trigger a conformational change in Efl1 analog to that ocurring to elongation factors during translation, promoting the release of the antiassociation factor Tif6 and allowing the 60S to enter the pool of active ribosomes in the cytoplasm. In this model, it has been suggested that Efl1 also assess the proper folding of the SRL. This loop of rRNA has been suggested to participate in activation of the GTPase activity (Voorhees et al., 2010, Koch et al., 2015, Clementi et al., 2010), but the exact mechanism of activation remains unclear and it is a matter of controversial debate because it is not fully compatible with certain data. It has been described how disruption of the SRL by α-sarcin or the ribosome inactivating protein PAP does not affect elongation factor-independent peptide synthesis (Chan and Wool 2008), and an intact SRL is not critical for GTP hydrolysis on EF-G or EF-Tu, but it is indispensable for stably binding of EF-G to the ribosome (Shi et al., 2012; García-Ortega 2010). Moreover, disruption of the SRL by α-sarcin differentially affects EF-G and EF-Tu binding to the ribosome (García-Ortega 2010), affecting significantly more the EF-G binding, GTP hydrolysis and translocation. Thus, the mechanism of GTP hydrolysis as well as binding to the ribosome might differ between translational GTPases, including Efl1. Our results indicate that cleavage of the SRL does not affect the localization of Tif6 and seems to not be affecting the general maturation pathway of the 60S subunit. It is possible then that the integrity of the SRL is not being checked during Tif6 release by Efl1.

References

Alford, S. C., Pearson, J. D., Carette, A., Ingham, R. J. and Howard, P. L. (2009). "Alpha-sarcin catalytic activity is not required for cytotoxicity." *BMC Biochem* **10**: 9.

Altvater, M., Chang, Y., Melnik, A., Occhipinti, L., Schutz, S., Rothenbusch, U., Picotti, P. and Panse, V. G. (2012). "Targeted proteomics reveals compositional dynamics of 60S pre-ribosomes after nuclear export." *Mol Syst Biol* **8**: 628.

Altvater, M., Schutz, S., Chang, Y. and Panse, V. G. (2014). "Dissecting ribosome assembly and transport in budding yeast." *Methods Cell Biol* **122**: 437-461.

Ben-Shem, A., Garreau de Loubresse, N., Melnikov, S., Jenner, L., Yusupova, G. and Yusupov, M. (2011). "The structure of the eukaryotic ribosome at 3.0 A resolution." *Science* **334**(6062): 1524-1529.

Benton, B. M., Eng, W. K., Dunn, J. J., Studier, F. W., Sternglanz, R. and Fisher, P. A. (1990). "Signal-mediated import of bacteriophage T7 RNA polymerase into the *Saccharomyces cerevisiae* nucleus and specific transcription of target genes." *Molecular and Cellular Biology* **10**(1): 353-360.

Bussiere, C., Hashem, Y., Arora, S., Frank, J. and Johnson, A. W. (2012). "Integrity of the P-site is probed during maturation of the 60S ribosomal subunit." *The Journal of Cell Biology* **197**(6): 747-759.

Chan, Y. L. and Wool, I. G. (2008). "The integrity of the sarcin/ricin domain of 23 S ribosomal RNA is not required for elongation factor-independent peptide synthesis." *J Mol Biol* **378**(1): 12-19.

Clementi, N., Chirkova, A., Puffer, B., Micura, R. and Polacek, N. (2010). "Atomic mutagenesis reveals A2660 of 23S ribosomal RNA as key to EF-G GTPase activation." *Nat Chem Biol* **6**(5): 344-351.

de la Cruz, J., Karbstein, K. and Woolford, J. L., Jr. (2015). "Functions of ribosomal proteins in assembly of eukaryotic ribosomes in vivo." *Annu Rev Biochem* **84**: 93-129.

Endo, Y. and Wool, I. G. (1982). "The site of action of alpha-sarcin on eukaryotic ribosomes. The sequence at the alpha-sarcin cleavage site in 28 S ribosomal ribonucleic acid." *J Biol Chem* **257**(15): 9054-9060.

Faza, M. B., Chang, Y., Occhipinti, L., Kemmler, S. and Panse, V. G. (2012). "Role of Mex67-Mtr2 in the nuclear export of 40S pre-ribosomes." *PLoS Genet* **8**(8): e1002915.

Freed, E. F., Bleichert, F., Dutca, L. M. and Baserga, S. J. (2010). "When ribosomes go bad: diseases of ribosome biogenesis." *Mol Biosyst* **6**(3): 481-493.

García-Mayoral, F., García-Ortega, L., Álvarez-García, E., Bruix, M., Gavilanes, J. G. and Martínez del Pozo, Á. (2005). "Modeling the highly specific ribotoxin recognition of ribosomes." *FEBS Letters* **579**(30): 6859-6864.

García-Mayoral, M. F., García-Ortega, L., Lillo, M. P., Santoro, J., Martínez del Pozo, A., Gavilanes, J. G., Rico, M. and Bruix, M. (2004). "NMR structure of the noncytotoxic alpha-sarcin mutant Δ(7-22): the importance of the native conformation of peripheral loops for activity." *Protein Sci* **13**(4): 1000-1011.

García-Ortega, L., Álvarez-García, E., Gavilanes, J. G., Martínez-del-Pozo, A. and Joseph, S. (2010). "Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding." *Nucleic Acids Res* **38**(12): 4108-4119.

García-Ortega, L., Masip, M., Mancheño, J. M., Oñaderra, M., Lizarbe, M. A., García-Mayoral, M. F., Bruix, M., Martínez del Pozo, A. and Gavilanes, J. G. (2002). "Deletion of the NH2-terminal beta-hairpin of the ribotoxin alpha-sarcin produces a nontoxic but active ribonuclease." *J Biol Chem* **277**(21): 18632-18639.

Gerhardy, S., Menet, A., Peña, C., Petkowski, J. and Panse, V. (2014). "Assembly and nuclear export of pre-ribosomal particles in budding yeast." Chromosoma 123(4): 327-344.

Greber, B. J., Boehringer, D., Godinic-Mikulcic, V., Crnkovic, A., Ibba, M., Weygand-Durasevic, I. and Ban, N. (2012). "Cryo-EM Structure of the Archaeal 50S Ribosomal Subunit in Complex with Initiation Factor 6 and Implications for Ribosome Evolution." *J Mol Biol* **418**(3–4): 145-160.

Herrero-Galán, E., Lacadena, J., Martínez-del-Pozo, A., Boucias, D. G., Olmo, N., Oñaderra, M. and Gavilanes, J. G. (2008). "The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin." *Proteins* **72**(1): 217-228.

Klinge, S., Voigts-Hoffmann, F., Leibundgut, M., Arpagaus, S. and Ban, N. (2011). "Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6." *Science* **334**(6058): 941-948.

Koch, M., Flur, S., Kreutz, C., Ennifar, E., Micura, R. and Polacek, N. (2015). "Role of a ribosomal RNA phosphate oxygen during the EF-G-triggered GTP hydrolysis." *Proc Natl Acad Sci* **112**(20): E2561-2568.

Lacadena, J., Álvarez-García, E., Carreras-Sangrà, N., Herrero-Galán, E., Alegre-Cebollada, J., García-Ortega, L., Oñaderra, M., Gavilanes, J. G. and Martínez-del-Pozo, A. (2007). "Fungal ribotoxins: molecular dissection of a family of natural killers." *FEMS Microbiol Rev* **31**: 212-237.

Lacadena, J., Martínez del Pozo, A., Barbero, J. L., Mancheño, J. M., Gasset, M., Oñaderra, M., López-Otín, C., Ortega, S., García, J. and Gavilanes, J. G. (1994). "Overproduction and purification of biologically active native fungal alpha-sarcin in *Escherichia coli*." *Gene* **142**(1): 147-151.

Lacadena, J., Martínez del Pozo, A., Martínez-Ruiz, A., Pérez-Cañadillas, J. M., Bruix, M., Mancheño, J. M., Oñaderra, M. and Gavilanes, J. G. (1999). "Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin alpha-sarcin." *Proteins* **37**(3): 474-484.

Lafontaine, D. L. J. (2015). "Noncoding RNAs in eukaryotic ribosome biogenesis and function." *Nat Struct Mol Biol* **22**(1): 11-19.

Masip, M., Lacadena, J., Mancheño, J. M., Oñaderra, M., Martínez-Ruiz, A., Martínez del Pozo, Á. and Gavilanes, J. G. (2001). "Arginine 121 is a crucial residue for the specific cytotoxic activity of the ribotoxin α-sarcin." *Eur J Biochem* **268**(23): 6190-6196.

Narla, A. and Ebert, B. L. (2010). "Ribosomopathies: human disorders of ribosome dysfunction." *Blood* **115**(16): 3196-3205.

Olmo, N., Turnay, J., González de Buitrago, G., López de Silanes, I., Gavilanes, J. G. and Lizarbe, M. A. (2001). "Cytotoxic mechanism of the ribotoxin α-sarcin. Induction of cell death via apoptosis." *Eur J Biochem* **268**(7): 2113-2123.

Olombrada, M., Martínez-del-Pozo, A., Medina, P., Budia, F., Gavilanes, J. G. and García-Ortega, L. (2014a). "Fungal ribotoxins: Natural protein-based weapons against insects." *Toxicon* **83**: 69-74.

Olombrada, M., Rodríguez-Mateos, M., Prieto, D., Pla, J., Remacha, M., Martínez-del-Pozo, A., Gavilanes, J. G., Ballesta, J. P. and García-Ortega, L. (2014b). "The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by α-sarcin of the eukaryotic ribosome." *Biochemistry* **53**(10): 1545-1547.

Olson, B. H. and Goerner, G. L. (1965). "Alpha sarcin, a new antitumor agent. I. Isolation, purification, chemical composition, and the identity of a new amino acid." *Appl Microbiol* **13**: 314-321.

Panse, V. G. and Johnson, A. W. (2010). "Maturation of eukaryotic ribosomes: acquisition of functionality." *Trends Biochem Sci* **35**(5): 260-266.

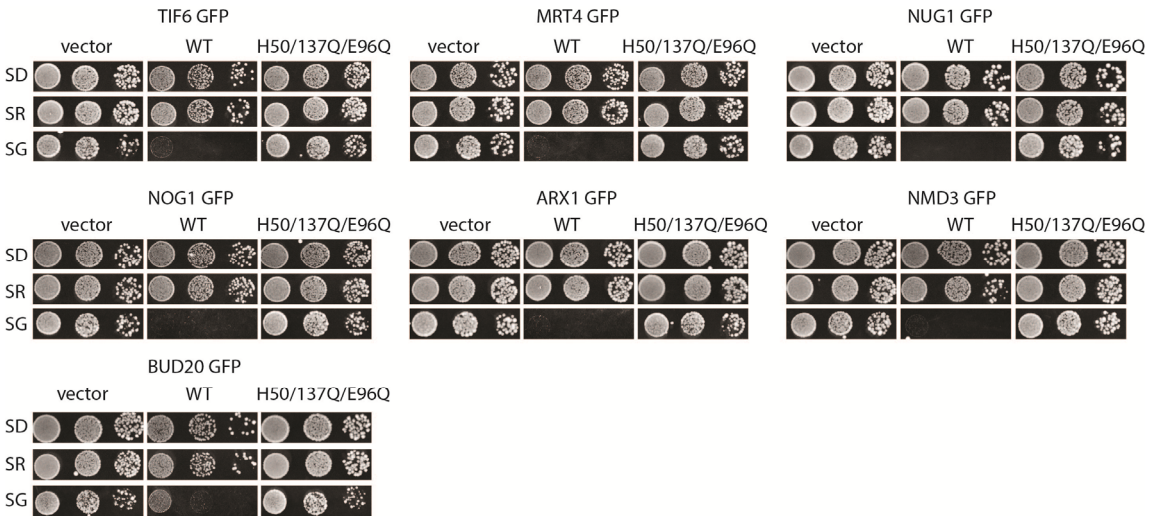
Schindler, D. G. and Davies, J. E. (1977). "Specific cleavage of ribosomal RNA caused by alpha sarcin." *Nucleic Acids Res* **4**(4): 1097-1110.

Shi, X., Khade, P. K., Sanbonmatsu, K. Y. and Joseph, S. (2012). "Functional role of the sarcin-ricin loop of the 23S rRNA in the elongation cycle of protein synthesis." *J Mol Biol* **419**(3-4): 125-138.

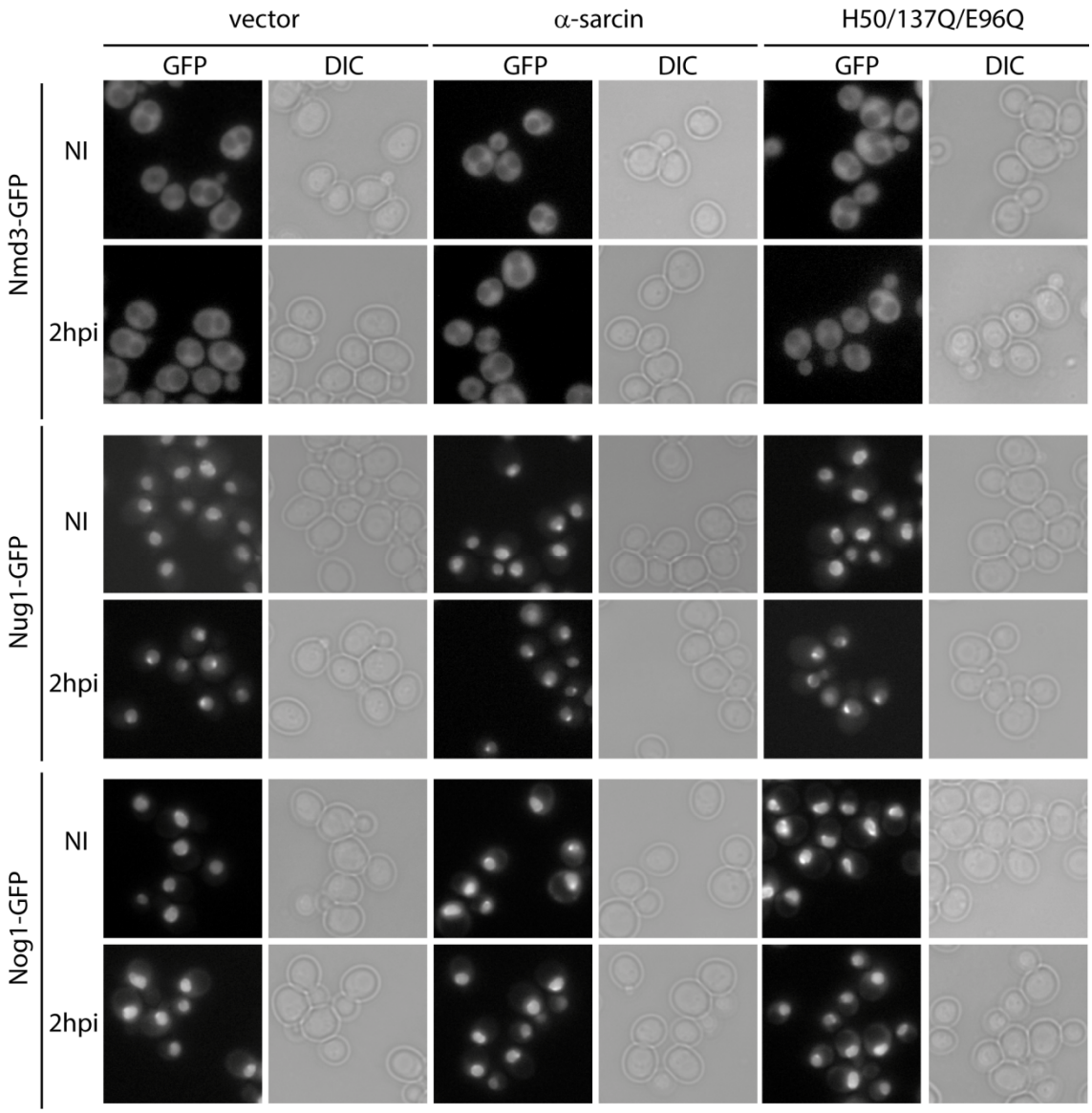
Voorhees, R. M., Schmeing, T. M., Kelley, A. C. and Ramakrishnan, V. (2010). "The Mechanism for Activation of GTP Hydrolysis on the Ribosome." *Science* **330**(6005): 835-838.

Woolford, J. L., Jr. and Baserga, S. J. (2013). "Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*." *Genetics* **195**(3): 643-681.

Supplementary Material



Supplementary figure 1.-Heterologous expression of ribotoxins in yeast. A) Yeast cells expressing different GFP-tagged trans-acting factors were transformed with plasmids containing α-sarcin (WT) or an inactive mutant (H50/137Q/E96Q) were spotted in 10-fold serial dilutions on glucose (SD), raffinose (SR) or galactose (SG) plates and grown at 30ºC for 3-6 days. Growth of yeast was inhibited when the expression of wild type α-sarcin was induced. Empty vector was used as a control.



Supplementary figure 2.-Recycling of trans-acting factors is not affected by α-sarcin expression (II). Yeast strains containing different GFP-tagged trans-acting factors (Nog1, Nug1, Nmd3) were transformed with plasmids containing α-sarcin or the catalytically inactive mutant H50/137Q/E96Q. Intracellular location of these factors was analyzed by fluorescence microscopy before (NI) and after 2 hours of induction (2hpi) of ribotoxin expression. Empty vector was used as control. Scale bar=5 μm.

Supplementary Table 1. Yeast strains used in this study.

Strain name	Genotype	Origin
BY4741	*MATa ura3 his3 leu2 met15 TRP1*	EUROSCARF
Ssf1-TAP	*MATa ura3 leu2 trp1 SSF1-TAP::HIS3MX*	Open biosystems
Rix1-TAP	*MATα ura3 his3 leu2 trp1 met15 RIX1-TAP::TRP1*	Open biosystems
Arx1-TAP	*MATa ura3 his3 leu2 met15 TRP1 ARX-TAP::HIS3MX*	Open biosystems
Kre35-TAP	*MATα ura3 his3 leu2 trp1 met15 KRE35-TAP::TRP1*	Open biosystems
Arx1-GFP	*MATa ura3 his3 leu2 met15 TRP1 ARX1-GFP::HIS3MX*	Open biosystems
Bud20-GFP	*MATa ura3 his3 leu2 met15 TRP1 BUD20-GFP::HIS3MX*	Open biosystems
Mrt4-GFP	*MATa ura3 his3 leu2 met15 TRP1 MRT4-GFP::HIS3MX*	Open biosystems
Nog1-GFP	*MATa ura3 his3 leu2 met15 TRP1 NOG1-GFP::HIS3MX*	Open biosystems
Nug1-GFP	*MATa ura3 his3 leu2 met15 TRP1 NUG1-GFP::HIS3MX*	Open biosystems
Tif6-GFP	*MATa ura3 his3 leu2 met15 TRP1 TIF6-GFP::HIS3MX*	Open biosystems
Nmd3-GFP	*MATa ura3 his3 leu2 met15 TRP1 NMD3-GFP::HIS3MX*	Open biosystems
bud20Δ	*MATa ura3 his3 leu2 met15 TRP1 bud20::KANMX*	Altvater et al. 2012
yrb2Δ	*MATα his3 leu2 met15 ura3 yrb2::KANMX*	Faza et al. 2012

Supplementary Table 2. Plasmids used in this study.

Plasmid	Relevant markers	Source
$YEP352_{GAL}$	PGAL1 2µm URA3	Benton et al. 1990
$YEP352_{GAL}$-sar	PGAL1-SAR 2µm URA3	This study
$YEP352_{GAL}$-Δ(7-22)	PGAL1-Δ(7-22) 2µm URA3	This study
$YEP352_{GAL}$-H50/137Q/E96Q	PGAL1- H50/137Q/E96Q 2µm URA3	This study
$YEP352_{GAL}$-H50Q	PGAL1- H50Q 2µm URA3	This study
$YEP352_{GAL}$-H50Q/137Q	PGAL1- H50Q/137Q 2µm URA3	This study
$YEP352_{GAL}$-R121Q	PGAL1- R121Q 2µm URA3	This study
pRS315-L5-GFP	RPL5-eGFP CEN LEU2	Altvater et al. 2012
pRS315-S2-GFP	RPS2-eGFP CEN LEU2	Altvater et al. 2012

Discusión

La elevada especificidad de las ribotoxinas, capaces de inactivar el ribosoma cortando un único enlace fosfodiéster de entre los más de 5400 que contiene, las hace especiales entre las ribonucleasas. El estudio exhaustivo de su estructura y actividad citotóxica frente a todo tipo de ribosomas permite comenzar a desarrollar productos de interés biotecnológico basados en ellas. Sus propiedades antitumorales ya se están explotando con éxito en forma de inmunotoxinas frente a cáncer de colon, por ejemplo. Si bien, su potencial insecticida hace que también sean óptimas candidatas para el diseño de nuevos biopesticidas. Los resultados que esta Memoria recoge se refieren, y siempre dentro de este contexto, a diferentes aspectos de las ribotoxinas.

En primer lugar, se profundiza en el estudio de las relaciones estructura-función tanto de la α-sarcina como de la HtA. La secuenciación del genoma de numerosos hongos ha permitido, además, describir por primera vez la existencia de otra ribotoxina semejante a HtA, haciendo que ésta deje de ser una excepción y se la pueda considerar como la representante de un nuevo grupo de ribotoxinas.

En segundo lugar, las propiedades insecticidas, a raíz de los resultados aquí presentados, parecen confirmar la que sería la función biológica de las ribotoxinas. No es arriesgado, por tanto, proponer el uso de éstas en el control de plagas. La descripción de la anisoplina como miembro de las ribotoxinas refuerza esta idea, ya que *Metarhizium anisopliae*, el hongo que la produce, ya se utiliza como agente de control biológico.

Por último, se ha intentado establecer el mecanismo de interacción de la α-sarcina con el ribosoma eucariota, descartando que ésta interaccione con las mismas estructuras ribosomales que utiliza la ricina. También se ha descrito por vez primera la actividad de la α-sarcina sobre partículas inmaduras pre-60S, lo que ampliaría notablemente la capacidad citotóxica de las ribotoxinas, así como nuestro conocimiento sobre el proceso de maduración ribosomal.

Relaciones estructura-función en las ribotoxinas

Desde el punto de vista estructural, la α-sarcina es una de las ribotoxinas más estudiadas, hasta el punto de poder asignar funciones concretas a residuos concretos (Lacadena et al. 2007) e incluso a átomos específicos (Perez-Cañadilllas et al. 2003). El análisis mutacional de ribotoxinas como la HtA, de menor tamaño e identidad de secuencia, permite establecer similitudes y/o diferencias entre miembros de la familia (Herrero-Galán et al. 2012a, Herrero-Galán et al. 2012b). En el primer apartado de resultados se presentan aspectos específicos de la estructura y función de las ribotoxinas α-sarcina y HtA. Mediante la caracterización de diferentes variantes mutantes de éstas se han intentado adjudicar papeles a diferentes residuos y bucles, como su implicación en la actividad catalítica o su participación en la interacción con membranas, por ejemplo. En concreto, se ha estudiado el papel de los bucles 2 y 3 de la α-sarcina, así como el del bucle 5 y el extremo amino terminal de la HtA.

Una primera conclusión corroboraría que en las interacciones que se establecen directamente entre el SRL y la α-sarcina, juegan un papel muy relevante las interacciones electrostáticas. El bucle 3 contiene una triada de lisinas (K111, K112 y K114) que parece estar involucrada en el reconocimiento específico del SRL, favoreciendo la formación del complejo enzima:sustrato (Plantinga et al. 2008). En la restrictocina, esta triada de lisinas (K110, K111, K113), en especial la K113, parece establecer contacto directo con la G prominente, ayudando a localizar el sustrato en el centro activo (Yang et al. 2001, Plantinga et al. 2008). En esta Tesis se ha estudiado el papel de estas lisinas en la α-sarcina, confirmando que se trata de una región imprescindible para la actividad catalítica de la ribotoxina, pero también se ha podido asignar una nueva funcionalidad a las lisinas 111 y 114, ya que participarían en el acercamiento de las vesículas lipídicas, facilitando su agregación. Además, según la estructura de la α-sarcina (PDB ID: 1DE3), la K114 forma parte de una red de interacciones con la Y48, un residuo del centro activo clave para la especificidad de la ribotoxina por su sustrato. El cambio de carga que se produce en el mutante K114E desestabilizaría esta interacción, que resulta esencial para la catálisis (Álvarez-García et al. 2006). En cuanto a la funcionalidad del bucle 2, no parece que la H82 esté jugando un papel esencial en la actividad enzimática de la α-sarcina, si bien, su sustitución por glutamina (H82Q) parece provocar una reorganización estructural que expondría regiones de la proteína capaces de interaccionar con lípidos, lo que explicaría su mayor capacidad de permeabilizar vesículas lipídicas. El bucle 2 es, por tanto, una de las regiones que parecen implicadas en la interacción con vesículas lipídicas. Se trata de un bucle muy poco conservado cuando se compara con la HtA. De hecho, no existe ningún residuo similar en la HtA, y el aminoácido equivalente al W50, con el que interacciona la H82 de la α-sarcina, es una arginina en la HtA. Dentro de este mismo estudio acerca del papel del bucle 2, la sustitución de los residuos 79 a 93 por los correspondientes residuos de HtA (ADAI) acorta notablemente la extensión de este

bucle, algo que parece afectar a la disposición geométrica del centro activo y da lugar a la pérdida completa de actividad enzimática. Los ensayos con lípidos demuestran que la eliminación de esta parte del bucle 2 no conlleva sin embargo un cambio en la funcionalidad de la toxina frente a vesículas lipídicas. Se puede entonces concluir que esta secuencia no estaría involucrada en la interacción de la α-sarcina con membranas.

El caso de la HtA es aún más interesante, no sólo porque es una ribotoxina menos estudiada sino, sobre todo, porque se trata de una proteína de tamaño intermedio entre las ribotoxinas y las RNasas no tóxicas del tipo de la RNasa T1. A pesar de este menor tamaño, la HtA mantiene la actividad ribonucleolítica específica de la α-sarcina frente al SRL y es también capaz de interaccionar con membranas lipídicas (Herrero-Galán et al. 2008). Todas las características de las ribotoxinas han tenido que acomodarse, por tanto, en una estructura más pequeña, con lo cual existe la posibilidad de que algunas regiones de la proteína hayan perdido o adquirido funcionalidades con respecto a las de la α-sarcina. Así pues, el estudio de mutantes de HtA contribuye a aclarar estas cuestiones. Como se ha comentado en la Introducción, una de las diferencias más obvias a la hora de comparar la α-sarcina y la HtA es la longitud de la horquilla β amino terminal. Una región que se sabe que es importante en la actividad ribonucleolítica, la interacción con membranas y el reconocimiento del ribosoma por parte de las ribotoxinas clásicas, las de mayor tamaño (García-Ortega et al. 2002). Esta horquilla β amino terminal no sólo es significativamente más corta sino que además posee menos cargas positivas que la de la α-sarcina (Figura D1). Por este motivo, se ha abordado el estudio de esta estructura y de la posibilidad de que el acortamiento de esta región se vea compensado por la mayor longitud y orientación del bucle 5 en la HtA, que además contiene varias lisinas susceptibles de sustituir a las que aparecen en la horquilla β amino terminal de la α-sarcina. Para ello se han producido y caracterizado diferentes variantes mutantes de HtA: el mutante de deleción Δ(8-15) y las mutaciones puntuales K115E, K118E y K123E que afectan a las lisinas anteriormente mencionadas. Al contrario de lo que ocurría al eliminar la horquilla de la α-sarcina, donde se perdía la capacidad de reconocer específicamente el ribosoma (García-Ortega et al. 2002), esta deleción da lugar en la HtA a una variante que sí mantiene la actividad ribonucleolítica tanto frente a ribosomas como frente al SRL aislado, si bien esta actividad es menor que la de la proteína silvestre. Ocurre lo mismo con el resto de mutaciones puntuales del bucle 5. A la hora de abordar este trabajo, ya se había demostrado cómo el centro activo de esta ribotoxina es menos rígido que el de la α-sarcina, dado que mutaciones puntuales en el centro activo de la HtA no producen la pérdida completa de la actividad catalítica específica de la enzima (Herrero-Galán et al. 2012a). Este resultado se consideró muy llamativo dado que la sustitución de los residuos equivalentes en la α-sarcina convertían a esta enzima en una RNasa completamente inactiva (Lacadena et al. 1999). Esta flexibilidad, sin embargo, parece convertir a la HtA en una enzima cuya especificidad depende más del contexto de las interacciones con su sustrato, ya que los mencionados mutantes sólo

son capaces de cortar el SRL cuando forma parte del ribosoma (Herrero-Galán et al. 2012a). Los resultados recogidos ahora en este apartado de la Memoria, en concordancia con estos resultados anteriores, sugieren que aún presentando la misma actividad, variaciones en el microentorno de los centros activos afectan de distinta manera a la HtA y la α-sarcina, presentando una mayor plasticidad la HtA, donde los cambios en su centro activo pueden ser compensados sin apenas pérdida de actividad específica. Así, la α-sarcina poseería un centro activo más rígido, donde la modificación de alguno de los residuos implicados en la catálisis da lugar a variantes completamente inactivas.

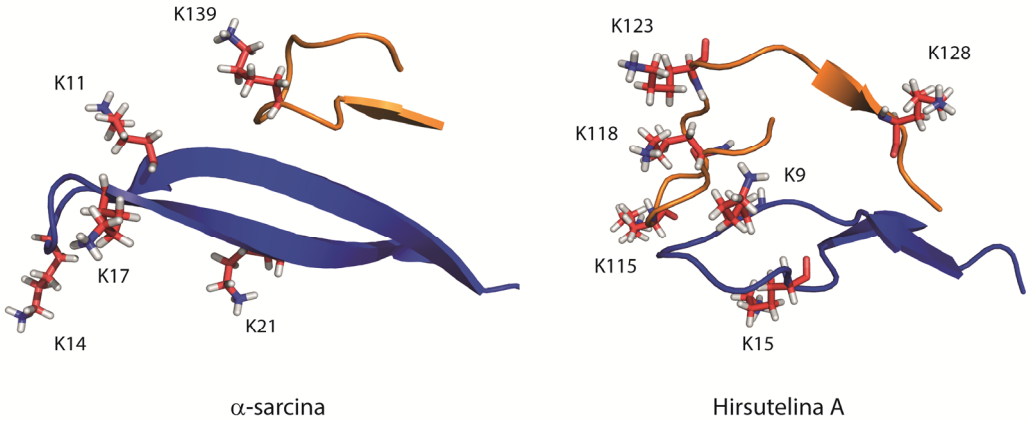


Figura D.1.-Comparación de la estructura de la horquilla β amino terminal (azul) y del bucle 5 (naranja) de la α-sarcina y de la HtA, destacando todas las lisinas que contienen. Ambos esquemas han sido generados con el programa PyMol.

Aunque en principio parece que la horquilla β amino terminal de la HtA no participaría en el reconocimiento del ribosoma, es posible que el bucle 5 pueda compensar en este sentido la deleción de dicha horquilla. Sería interesante comprobar el comportamiento de una variante donde se eliminasen ambas estructuras (bucle 5 y horquilla β amino terminal), para poder determinar su función. Las lisinas estudiadas podrían estar supliendo la falta de cargas positivas de la horquilla, ya sea para el reconocimiento del ribosoma o en la interacción con membranas celulares. Este último aspecto no se ha abordado en la Memoria, pero debido a su orientación en la estructura de la HtA y a raíz de los resultados de citotoxicidad frente a células de insecto, donde el mutante K123E presenta una IC_{50} unas 10 veces menor que la HtA silvestre, la K123, parece ser un buen candidato para establecer estas interacciones con lípidos.

Caracterización de una nueva ribotoxina: la anisoplina de *Metarhizium anisopliae*

Hasta la fecha, la mayor parte de las ribotoxinas fúngicas bien caracterizadas se han descrito en hongos del género *Aspergillus*. Si bien, el descubrimiento de que el hongo entomopatógeno *Hirsutella thompsonii* producía la ribotoxina HtA abrió la posibilidad de que esta familia de proteínas estuviera más extendida de lo que se

pensaba inicialmente. Las técnicas de secuenciación genómica han puesto a disposición de la comunidad científica el genoma completo de multitud de organismos, entre ellos un número creciente de hongos (Galagan et al. 2005, Oka et al. 1990), permitiendo, mediante la comparación de sus secuencias, la búsqueda de nuevas ribotoxinas. Se ha analizado la posible presencia de genes que codificaran para ribotoxinas en el hongo entomopatógeno *Metarhizium anisopliae*, de especial interés en biotecnología. El género *Metarhizium* se distribuye por todo el planeta, desde el ártico a los trópicos. Algunas especies, como *M. acridum*, son altamente específicas de especie, mientras que otras como *M. anisopliae* poseen una especificidad menos restringida, resultando patógeno para más de 200 especies de insectos, entre ellos el mosquito portador de la malaria. Este hongo contiene más genes en su genoma que otros de la misma especie, lo que le hace más versátil y, probablemente, explique la elevada variedad de especies que afecta (Oka et al. 1990). Esta característica convierte a *M. anisopliae* en un perfecto candidato para su uso como agente para el control biológico. El análisis del genoma de *M. anisopliae* reveló la existencia de una proteína semejante a la HtA que compartía un 70% de identidad de secuencia con ella (Figura A3.1). Esta potencial ribotoxina se bautizó como anisoplina, nombre que abreviamos como Anp. Además, el alineamiento de secuencias indica que existen proteínas similares a ésta (95-99% de identidad de secuencia, resultados no mostrados en esta Memoria) en otras especies de *Metarhizium*. El análisis del medio de cultivo de *M. anisopliae* demostró la presencia de la actividad ribonucleolítica específica de las ribotoxinas, ya que se observaba la liberación de fragmento α característico al ensayar distintas alícuotas del medio frente a un lisado de reticulocitos (Figura A3.1). No obstante, no se detectó ninguna proteína en el medio extracelular a la que responsabilizar de esta actividad, probablemente porque en las condiciones de laboratorio empleadas se produce en cantidades mínimas.

Se optó entonces por la producción de la proteína de forma recombinante, con el mismo planteamiento que el utilizado para la α-sarcina (Garcia-Ortega et al. 2000). Se purificó a homogeneidad una proteína del tamaño esperado cuya caracterización estructural reveló muy pocos cambios en comparación con la estructura de la HtA. Lo más relevante, quizás, fueron los datos de emisión de fluorescencia, que sugerían una mayor contribución de los triptófanos de la Anp, pese a estar conservados con respecto a la HtA. Algo que se ha atribuido a un diferente microentorno de estos residuos en la estructura tridimensional de la proteína. Los programas de predicción de estructura de proteínas Phyre2 y SWISS-MODEL sugieren que la Anp posee la misma estructura tridimensional que la HtA, con pocos cambios en los elementos de estructura secundaria (Figura D.2).

La caracterización funcional de la Anp se centró en la detección de la actividad ribonucleolítica específica de las ribotoxinas. De acuerdo con este criterio, Anp se comportó como una ribotoxina frente a todos los sustratos ensayados, con una actividad citotóxica comparable a la de HtA, como se deduce de los valores de IC_{50} en

los ensayos frente a células de insecto (Figura A3.3). Las mayores diferencias, aunque siguen siendo pequeñas, se encontraron a la hora de ensayarla frente a ribosomas completos aislados, donde la Anp sería unas 50 veces menos activa que la HtA, sugiriendo una peor interacción con el ribosoma (Figura A3.3). Una observación que se puede explicar recurriendo a las interacciones electrostáticas, muy importantes a la hora de explicar el reconocimiento ribosomal por parte de las ribotoxinas. De este modo, el punto isoeléctrico teórico de la Anp es de 8.3 en lugar de 9.3 que es el de la HtA. Más aún, comparando ambas secuencias polipeptídicas, las principales diferencias aparecen en el extremo amino terminal, siendo más largo y con carácter ácido el de la Anp. En cualquier caso, las diferencias que aparecen entre la HtA y la Anp en cuanto a su actividad sobre ribosomas son similares a las que se dan entre la HtA y la α–sarcina (Figura B1.5) y, teniendo en cuenta la importancia de la región amino terminal en la funcionalidad de las ribotoxinas y en el reconocimiento ribosomal, los resultados obtenidos con Anp entran dentro de lo esperable considerando la variabilidad en secuencia y función que se observa dentro de la familia de ribotoxinas. Quedaría entonces por estudiar sus propiedades frente a lípidos, aunque todo parece indicar que su comportamiento será similar al de la HtA.

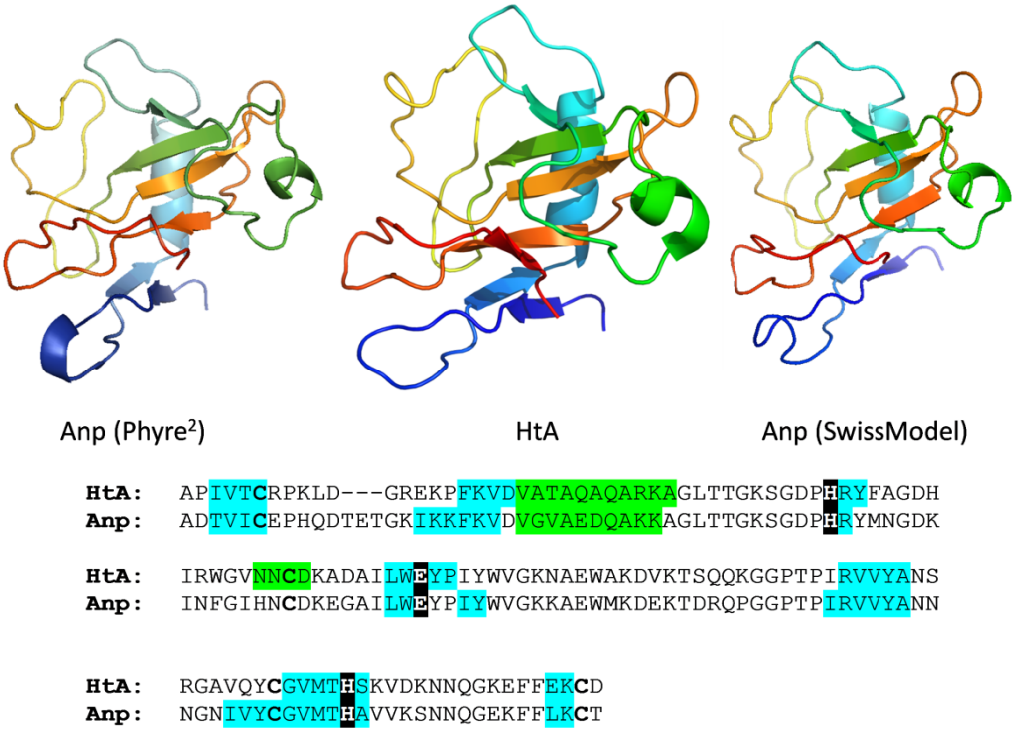


Figura D.2.-Modelo de la estructura tridimensional de la Anp utilizando el software Phyre[2] (izquierda) o SwissModel (derecha), y estructura tridimensional de la HtA (centro) y alineamiento de secuencias donde se indica la predicción de estructura secundaria obtenida con Phyre[2] (α-helice, verde; lámina β, azul) y los residuos catalíticos (negro).

Los resultados demuestran, en definitiva, que la Anp se comporta como una ribotoxina cuyas características se asemejan más a la HtA que al resto de ribotoxinas. Este descubrimiento resulta interesante puesto que se pensaba que HtA era una

excepción dentro de esta familia. La descripción de la Anp como una ribotoxina sugiere la posibilidad de que HtA no sea tan excepcional como parecía, sino más bien que forme parte de un subgrupo de ribotoxinas. El estudio pormenorizado del genoma de otros hongos distintos a *Metarhizium* probablemente revele la existencia de nuevas ribotoxinas, apoyando la idea de que este grupo de toxinas está más extendido de lo que se pensaba hasta ahora. Llama la atención que estas dos ribotoxinas, HtA y Anp, sean producidas por hongos entomopatógenos, mientras que el resto están producidas por hongos del género *Aspergillus*. Quizás estos dos tipos de hongos hayan utilizado las ribotoxinas con diferentes objetivos, adaptando su estructura y funcionalidad en cada caso; así, mientras que *Aspergillus* parece que las utiliza como defensa frente a depredadores durante la maduración de los conidios, puede que los hongos entomopatógenos como *Hirsutella* o *Metarhizium* las utilicen como un factor de virulencia frente a los insectos que parasitan. El descubrimiento de nuevas ribotoxinas como ésta es, en cualquier caso, muy interesante desde el punto de vista biotecnológico, ya que puede tratarse de un factor de virulencia frente a insectos con potencial uso como insecticida, como se comentará más adelante.

Toxinas en la defensa frente a depredadores

Las ribotoxinas se describieron por primera vez como agentes antitumorales. Eran eficaces frente a sarcomas y carcinomas inducidos en ratones; sin embargo, su citotoxicidad resultó ser demasiado inespecífica, lo que limitó su uso terapéutico (Olson and Goerner 1965, Roga et al. 1971). Durante los últimos 30 años, esta familia de toxinas se ha estudiado a nivel molecular y, actualmente, se conoce en gran detalle su estructura y mecanismo citotóxico (Lacadena et al. 2007). Ello ha permitido retomar su estudio como agente antitumoral, esta vez como dominio tóxico de inmunotoxinas (Carreras-Sangrà et al. 2012, Tome-Amat et al. 2015a). Estas quimeras ya han demostrado con éxito su especificidad y eficacia frente a xenotransplantes de cáncer de colon en ratones atímicos (Tome-Amat et al. 2015c).

Obviamente, su aplicación terapéutica como agente antitumoral, está lejos de su función biológica. En su ambiente natural, los hongos productores de ribotoxinas se encuentran bajo amenaza constante de fungívoros tales como colémbolos, ácaros o insectos (Berenbaum and Eisner 2008). Una de las estructuras más vulnerables al ataque de dichos depredadores serían los conidios, por el hecho de ser los encargados de la reproducción y propagación del hongo. De hecho, parece que las ribotoxinas se localizan específicamente en las fiálides de los conidios (Figura I.3, Brandhorst and Kenealy 1992), donde se detectan durante el proceso de maduración de estas estructuras reproductoras, desapareciendo una vez termina ésta. En este sentido, las ribotoxinas ejercerían una función de defensa en los conidios, y una vez degradadas, los depredadores serían capaces de alimentarse del hongo y transportar y diseminar las esporas a otros lugares. Por tanto, se podría considerar a las ribotoxinas como proteínas con función insecticida, ya que la mayor parte de los depredadores de estos

hongos son insectos. Dicha asunción viene apoyada además por los primeros estudios realizados con la ribotoxina HtA. Esta toxina fue identificada en primer lugar en filtrados del hongo entomopatógeno *Hirsutella thompsonii* como una proteína de origen fúngico capaz de actuar sobre larvas de *Galleria mellonella* y *Aedes aegypti* (Liu et al. 1995, Mazet and Vey 1995), describiéndose después como una ribotoxina capaz de inactivar el ribosoma (Liu et al. 1996, Herrero-Galán et al. 2008).

Ahora se ha explorado la posibilidad de que la función insecticida de la HtA no fuese un caso aislado, sino que constituyera la principal función de las ribotoxinas en el contexto biológico del hongo. Con esa intención, se realizó un estudio comparativo entre la HtA, con actividad insecticida previamente demostrada, y la α-sarcina, una de las ribotoxinas más representativas (Resultados B1, Olombrada et al. 2013). Los estudios demostraron la toxicidad de ambas proteínas frente a larvas de *Galleria mellonella* (Figura B1.1) y cultivos de células de insecto (Figuras B1.2 y B1.3), casi con la misma eficacia y una toxicidad dos órdenes de magnitud por encima de la que presentan frente a células tumorales humanas. Las mayores diferencias entre ellas se observaron al ensayar su actividad frente a ribosomas de insecto aislados. En ese caso lo que se evalúa es exclusivamente la capacidad de corte del SRL, sin tener en cuenta la habilidad de las toxinas de atravesar membranas lipídicas. En estos ensayos, y tal y como se ha comentado antes, la HtA resulta significativamente más activa que la α-sarcina (Figura B1.5). Estos resultados confirman una característica funcional de las ribotoxinas que ya se sospechaba, que el paso a través de las membranas fuese un paso limitante en su citotoxicidad. De hecho, algo similar ocurre al comparar la actividad ribonucleolítica sobre los ribosomas y la citotoxicidad de la HtA y la Anp (Figura A3.3). Por otro lado, podría ocurrir que el mecanismo por el cual la α-sarcina y HtA contactan con las membranas lipídicas sea diferente, como evidencia su distinto comportamiento frente a vesículas modelo (Gasset et al. 1989, Gasset et al. 1990, Herrero-Galán et al. 2008). No obstante, hay que tener en cuenta que en el medio natural hay muchos otros factores que también pueden modular la actividad de las ribotoxinas, como la velocidad de síntesis y de transporte al medio extracelular o la estabilidad de la proteína una vez secretada. *Aspergillus*, productor de α-sarcina, e *Hirsutella*, productor de HtA, aparecen en ambientes diferentes, y por tanto la actividad de sus ribotoxinas podría no ser directamente comparable. Los resultados recogidos en esta Tesis, sin embargo, demuestran que ambas toxinas son muy eficientes frente a insectos, apoyando la idea de que su función en el medio natural es la de defender al hongo frente a insectos depredadores o la de contribuir al establecimiento de relaciones de parasitismo con ellos. La caracterización de la nueva ribotoxina Anp de *Metarizhium anisopliae* ha demostrado que esta toxina también mantiene su actividad frente a células de insecto con la misma eficiencia con que lo hace la HtA, lo que refuerza esta hipótesis.

Desarrollo de nuevos biopesticidas basados en las ribotoxinas

Los biopesticidas se encuentran entre las nuevas estrategias para el control de plagas de insectos y otros patógenos. Son una buena alternativa a los agentes químicos, que presentan problemas como la creciente resistencia y la toxicidad (Glare et al. 2012). Los hongos entomopatógenos como *H. thompsonii* juegan un papel fundamental en el control natural de plagas de insecto. De hecho, en conjunto, este tipo de hongos puede atacar a casi cualquier especie de insecto conocida, aunque individualmente son bastante específicos de especie. Algunos de ellos incluso ya se están comercializando como agentes de control biológico o biopesticidas. Es el caso de *Beauveria* o *Metarhizium* (Kim et al. 2014, Faria and Wraight 2007), o el propio hongo productor de la HtA, *H. thompsonii*, que en algunos casos ha demostrado ser más eficaz que los anteriores (Rossi-Zalaf and Alves 2006). Sin embargo, todos ellos presentan limitaciones en entornos naturales, fuera del control del laboratorio, sobre todo en su especificidad, distribución, vida media y eficacia en el campo (Sreerama Kumar and Singh 2008). Para superar dichas limitaciones, se han abordado distintas estrategias. Una de ellas es la modificación genética del hongo para aumentar su virulencia o su espectro de acción, si bien, el uso de organismos modificados genéticamente (OMG) no está del todo extendido debido a la negativa imagen pública acerca de este tipo de organismos. Otra opción es la modificación genética de los propios cultivos, haciendo que expresen factores de virulencia contra insectos u otras plagas. Un ejemplo de ello es el arroz transgénico que expresa la proteína AFP, una proteína antifúngica secretada por *Aspergillus giganteus* junto a la ribotoxina α-sarcina. En este caso, el arroz presenta resistencia a algunos de los hongos filamentosos más patógenos, como *Magnaporte grisae* (Moreno et al. 2005). De nuevo, la reticencia en el uso de OMG está frenando la implantación de este tipo de cultivos.

El conocimiento del mecanismo de infección que utilizan los hongos ha permitido también aislar y caracterizar metabolitos tóxicos de los hongos con la intención de usarlos de manera individual o en combinación con otros como biopesticidas, e incluso modificarlos para desarrollar fórmulas mejoradas. Todo el conocimiento acumulado sobre la estructura y función de las ribotoxinas fúngicas se podrían utilizar para el desarrollo de biopesticidas de este tipo. Las ribotoxinas α-sarcina y HtA ya se están estudiando con la intención de utilizarse en el control de plagas. En colaboración con el grupo de Manejo Integrado de Plagas de la Universidad Politécnica de Madrid se están poniendo a punto ensayos de toxicidad de las ribotoxinas, administrando éstas por vía oral o tópica a especies de insectos como *Spodoptera exigua* y *Ceratitis capitata* (Tabla D.1). Hasta ahora no se han obtenido resultados satisfactorios, ya que la administración de ribotoxinas aisladas no afecta a la mortalidad ni a la fertilidad de los insectos. Es probable que el método de administración no sea el adecuado o que la toxina sola no sea suficiente para inducir la

muerte del insecto. En el medio natural, el hongo entomopatógeno utiliza un cóctel de metabolitos insecticidas, entre los que se incluyen enzimas capaces de degradar la cutícula del insecto para poder invadirlo y crecer en su interior. Por ello, los biopesticidas que combinan varios metabolitos tóxicos resultarían más eficaces.

No hay que olvidar que las ribotoxinas fúngicas pueden inactivar cualquier tipo de ribosoma, con lo que su uso como biopesticidas estaría restringido debido a la posible citotoxicidad en humanos. Este problema podría solucionarse utilizando variantes mutantes que sean incapaces de atravesar las membranas de células humanas o, alternativamente, se puede recurrir al uso de baculovirus silvestres. Su uso ya se está aplicando con otros sistemas y parece muy prometedor debido a su especificidad frente a insectos. Más aún, los baculovirus recombinantes diseñados para aumentar su virulencia resultan una mejor alternativa (Cory et al. 1994, Inceoglu et al. 2001, Gramkow et al. 2010, Shim et al. 2013). Sería interesante utilizar este tipo de vector para la administración de ribotoxinas. El gen de la α-sarcina, la HtA o cualquier otra ribotoxina, o variante mutante de éstas, se podría introducir en el genoma del baculovirus, de modo que la toxina sólo se expresase durante la infección del insecto (Figura I.5). De este modo se conseguirían simultáneamente dos objetivos distintos: aumentar la virulencia del virus, ya que la toxina también sería letal para el insecto, y evitar la citotoxicidad de ésta frente a otros organismos.

Compuesto	Concentración (mg/L)	Fertilidad[1] (huevos/hembra y día)	Fertilidad (%) a los 4 días[2]	Fertilidad (%) a los 6 días[3]	Mortalidad (%) a las 72h
Tampón	0	25.57±2.60	78.18±4.14	59.52±2.46	0
α-sarcina	2000	22.03±4.03	79.05±3.41	66.43±3.18	0
Hirsutelina A	1000	27.43±3.98	82.75±3.17	67.14±3.37	0
***Beauveria bassiana** (control positivo)**	28.75	25.05±2.83	81.90±4.41	63.89±3.16	0
Lambda-cyhalotrin (control negativo)**	20	-	-	-	100

[1]F=0.43 df=3.24 P=0.7360

[2]F=0.80 df=3.24 P=0.8020

[3]F=1.26 df=3.24 P=0.3089

*Naturalis-L (*Beauveria bassiana* 2.3% ($2.3x10^6$ viable conidia/mL). Máxima dosis comercial empleada.

**Karate Zeon (Lambda cyhalotrin 10%). Máxima dosis comercial empleada.

Tabla D.1.-Mortalidad y fertilidad de *Ceratitis capitata* tras ingerir diferentes ribotoxinas fúngicas incluidas en la dieta.

Dado que *H. thompsonii* ha resultado ser eficaz en el tratamiento frente al ácaro *Varroa destructor* (Kanga et al. 2002, Peng et al. 2002, Shaw et al. 2002), uno de los factores implicados en el colapso de las colonias de abejas de la miel (*Apis mellifera*), que actualmente constituye un grave problema para los países occidentalizados (Evans and Schwarz 2011), se contempla también la posibilidad de utilizar las propiedades acaricidas de las ribotoxinas. La abeja de la miel no sólo tiene interés comercial, sino que resulta imprescindible, ya que estos insectos son polinizadores naturales de los cultivos, lo que aumenta la necesidad de combatir esta

enfermedad. Puesto que algunos baculovirus son capaces de atacar especies de la subclase *Acari*, podría resultar interesante el desarrollo de virus recombinantes que expresasen HtA y analizar su efecto sobre los ácaros y la viabilidad de las abejas.

En esta Tesis Doctoral se ha descrito una nueva ribotoxina, la Anp, producida por *M. anisopliae*. Dicho hongo se ha utilizado como agente de control biológico, destacando su actividad frente al mosquito de la malaria *Anopheles gambiae* (Whetstone and Hammock 2007). La principal estrategia en la lucha contra la malaria es la utilización de insecticidas frente al mosquito que transmite el parásito que la produce (*Plasmodium spp.*). La Organización Mundial de la Salud ya alertó en 2012 de que el abuso de insecticidas estaba causando la aparición de mosquitos resistentes, lo que podría acarrear graves consecuencias para la salud pública. Por ello resulta de vital importancia el desarrollo de nuevas estrategias de control biológico frente a estos mosquitos. En concreto, la ribotoxina Anp podría ser un factor de virulencia importante, y su aprovechamiento como biopesticida, ya sea sólo o en combinación con otras moléculas, o formando parte de baculovirus, se presenta como una oportunidad para controlar la población de *Anopheles* y así tratar de combatir la transmisión del parásito que causa la malaria.

Mecanismo de interacción de las ribotoxinas con el ribosoma eucariota

La elevada especificidad que presentan las ribotoxinas por un único enlace del ribosoma lleva a pensar que deben establecer una serie de interacciones con éste que las guíen de manera eficiente hacia el SRL. Una vez internalizadas en la célula, la naturaleza básica de las ribotoxinas les permitiría encontrar rápidamente el ribosoma, de naturaleza ácida. La toxina difundiría entonces por su superficie hasta localizar el SRL. La importancia de las interacciones electrostáticas que se establecen inicialmente con el ribosoma se han estudiado en profundidad (Korennykh et al. 2006, Korennykh et al. 2007, Plantinga et al. 2008, Plantinga et al. 2011), demostrando ser claves para el reconocimiento y adecuado corte del SRL, como se ha comentado anteriormente. De hecho, la velocidad con la que la α-sarcina hidroliza el ribosoma completo es unas 1000 veces superior a la que muestra con análogos del SRL aislados (Korennykh et al. 2006, Endo et al. 1988). Pero no parece factible que el reconocimiento del SRL por parte de las ribotoxinas dependa exclusivamente de las interacciones electrostáticas, sino que es probable que se establezcan contactos más específicos con regiones del ribosoma que las ayuden en su reconocimiento específico. En este contexto, ya se ha predicho el establecimiento de contactos entre la α-sarcina y algunas proteínas ribosomales (García-Mayoral et al. 2005). Mediante la modelización de la interacción que tendría lugar entre la α-sarcina y el ribosoma de *Haloarcula marismortui*, se predijo que la horquilla β amino terminal establecería contactos con la proteína ribosomal uL14, mientras que el bucle 2 lo haría con la proteína ribosomal uL6. Sin embargo, no es descartable que se produzcan interacciones adicionales con otros

elementos ribosomales, o que estas interacciones sean distintas en el caso de otras ribotoxinas diferentes.

Las proteínas inactivantes del ribosoma (RIPs), sin ir más lejos, comparten diana con las ribotoxinas a pesar de que no existen similitudes entre ellas, ni en su secuencia, ni en su estructura. La ricina, una RIP que se produce en las semillas de la planta del ricino (*Ricinus communis*), es una de las toxinas más potentes que se conocen, considerándose como una potencial arma biológica. Esta familia de toxinas se caracteriza por su actividad N-glicosidasa sobre una adenina del SRL conservada universalmente (A_{4324} del rRNA 28S de rata, A_{2660} del rRNA 23S de *E.coli*), precisamente la adenina adyacente al enlace fosfodiéster que corta la α-sarcina (G_{2661}-A_{2662}). Como sucede con las ribotoxinas, las interacciones electrostáticas juegan un papel importante en el reconocimiento inicial del SRL por parte de las RIPs (Korennykh et al. 2007). No obstante, para las RIPs ya se ha demostrado la existencia de interacciones específicas con ciertas estructuras del ribosoma. Así, se ha descrito la interacción entre la proteína antiviral del carmín (Pokeweed Antiviral Protein, PAP) y la proteína ribosomal L3 (Hudak et al. 1999), y también la interacción de RIPs como la ricina o la toxina de Shiga 1 (Stx1) con las proteínas del tallo ribosómico (Chiou et al. 2008, McCluskey et al. 2008). Este tallo ribosómico es una protuberancia de la subunidad mayor del ribosoma encargada de reclutar factores de elongación durante la traducción y está implicado en la activación de la actividad GTPasa de los factores EF-G y EF-Tu (Mohr et al. 2002, Gonzalo and Reboud 2003, Diaconu et al. 2005). Está formado por una proteína central, uL10, a la que se anclan dímeros de proteínas ácidas (P1/P2 en eucariotas, bL12 en procariotas). En particular, el tallo ribosómico de *S. cerevisiae* consta de dos heterodímeros (P1α/P2β-P1β/P2α) que se anclan a uL10 a través de su extremo amino terminal (Guarinos et al. 2001). La proteína uL10 es esencial para la viabilidad de la levadura (Santos and Ballesta 1994), mientras que la deleción de una o varias de las proteínas ácidas P1 y P2 da lugar a cepas viables de levadura; eso sí, con una velocidad de crecimiento reducida (Remacha et al. 1995). El tallo ribosómico es una estructura muy dinámica donde las proteínas ácidas P1/P2 no están siempre unidas al ribosoma. Existe un reservorio de estas proteínas en el citosol que sólo se unen a ribosomas completos 80S y polisomas. El ensamblaje de este tallo es, además, una de las últimas etapas del proceso de biogénesis y maduración de los ribosomas. Una vez terminada la traducción, los heterodímeros se desensamblan y vuelven a encontrarse libres en el citosol (Bautista-Santos and Zinker 2014), repitiéndose el ciclo. Volviendo a la interacción de las RIPs con el tallo ribosómico, parece que éstas siguen un modelo de unión en dos etapas: en primer lugar, a través de interacciones electrostáticas no específicas las moléculas de ricina se concentran en la superficie del ribosoma y, en una segunda etapa, contactan entonces con las proteínas ácidas del tallo ribosómico, que son las encargadas de aproximar la toxina y orientar su centro activo hacia el SRL (Chiou et al. 2008, Li et al. 2009, May et al. 2012,

Li et al. 2013, May et al. 2013). Además, parece que la ricina tiene más afinidad por uno de los dos heterodímeros P1/P2 que forman el tallo (Grela et al. 2014).

Se ha descrito la interacción de varias RIPs con el tallo ribosómico, si bien, no todos los miembros de esta familia de toxinas lo hacen. Cabe destacar que algunas RIPs sólo inactivan ribosomas eucariotas (ricina), mientras que otras inactivan ribosomas tanto eucariotas como procariotas (PAP), por lo que se ha propuesto que existan diferentes mecanismos de interacción con el ribosoma, dando así lugar a las distintas especificidades de cada toxina. Parece que la interacción con el tallo ribosómico ha sido un mecanismo de evolución convergente adoptado por algunas RIPs (Lapadula et al. 2012). Los resultados de esta Memoria indican que la ribotoxina α-sarcina, sin embargo, no utiliza el tallo ribosómico para interaccionar con el ribosoma y aproximarse al SRL (Resultados C1; Olombrada et al. 2014b). Los experimentos realizados tanto *in vivo* como *in vitro* prueban que el corte de este bucle de RNA por parte de la α-sarcina se produce tanto en presencia como en ausencia de las proteínas ácidas del tallo. Por tanto, todo parece indicar que la α-sarcina sigue un mecanismo alternativo de interacción con el ribosoma. No es descartable que dicho mecanismo sea similar al de la PAP, una RIP que también inactiva ambos tipos de ribosomas, por lo que sería interesante estudiar la posible interacción de la α-sarcina con la proteína ribosomal uL3. Además, dadas las distintas estructuras mostradas por ribotoxinas como la α-sarcina y la HtA y los diferentes resultados mostrados en esta Tesis a la hora de inactivar ribosomas por parte de la α-sarcina, la HtA y la Anp (Figuras A3.3 y B1.5), resultaría interesante también comparar el mecanismo de interacción de estas tres ribotoxinas con el ribosoma, ya que podrían existir diferencias.

La maduración del ribosoma como nueva diana de las ribotoxinas

En general, el proceso de ensamblaje de los ribosomas es complejo para la célula y muy costoso en términos energéticos. Una levadura, por ejemplo, necesita sintetizar alrededor de 200.000 ribosomas para poder dividirse. El estudio de la ruta de biogénesis del ribosoma durante los últimos 20 años ha permitido conocerla con detalle. En ella participan más de 300 factores, entre proteínas y RNAs (Woolford and Baserga 2013, Gerhardy et al. 2014, de la Cruz et al. 2015). Como ya se ha descrito en la Introducción, el ensamblaje de las subunidades ribosomales comienza en el nucleolo, pero prosigue en el nucleoplasma y en el citosol, hasta que se forma la partícula funcionalmente activa y madura.

Hasta ahora, la actividad de las ribotoxinas fúngicas sólo se había estudiado en ribosomas maduros. Puesto que las partículas pre-60S también contienen rRNA susceptible de ser atacado por las ribotoxinas, en el trabajo recogido en esta Memoria se pretendió estudiar si estos ribosomas aún inmaduros también podían ser su diana. Para ello se utilizó la levadura *S. cerevisiae* como organismo modelo, ya que el proceso de biogénesis del ribosoma en levaduras se conoce en gran detalle. Para poder cortar partículas pre-60S en estadíos tempranos de maduración, la α-sarcina debería ser

capaz de acceder al núcleo de la levadura. Por su pequeño tamaño, es posible que esta ribotoxina pudiese pasar a través del poro nuclear simplemente por difusión. La microscopía de fluorescencia reveló que la α-sarcina fusionada a GFP estaba efectivamente dispersa por toda la célula, incluido el núcleo, indicando que sí podía tener acceso a las mencionadas pre-60S tempranas, algo que ya se había planteado previamente (Alford et al. 2009). Durante estas primeras etapas de la maduración, el rRNA 25S no está del todo procesado, de modo que las partículas pre-60S nucleares tempranas contienen sobre todo pre-rRNA 27S. Los resultados que se presentan en esta Tesis sugieren que dicha forma de pre-rRNA no puede ser cortada por la α-sarcina (Figura C2.1). En conjunto, todo parece indicar que esta ribotoxina actúa principalmente sobre partículas pre-60S citoplásmicas a pesar de ser capaz de acceder al interior nuclear. La purificación de partículas pre-60S en distintos estadíos de maduración utilizando la técnica de purificación de afinidad en tándem (TAP, del inglés *Tandem Affinity Purification*) confirmó esta observación, puesto que la liberación del fragmento α era más evidente en partículas pre-60S tardías como las asociadas a Kre35-TAP o Arx1-TAP, mientras que apenas se detectó en las tempranas como las que se purifican unidas a Ssf1-TAP o Rix1-TAP y que están enriquecidas en pre-rRNA 27S (Figura C2.1). A primera vista, resulta muy interesante que la α-sarcina pueda discernir entre rRNA 25S y pre-rRNA 27S. Asumiendo que es capaz de entrar en el núcleo celular, como así parece, se plantea la cuestión de si el SRL no estará aún estructurado en las moléculas de pre-rRNA 27S o si se trata más bien de un problema de accesibilidad de la α-sarcina, debido a la gran cantidad de factores de ensamblaje que forman las partículas pre-60S tempranas. En este sentido, el pre-rRNA 27S contiene una larga secuencia espaciadora ITS1 que es eliminada durante su procesamiento a rRNA 25S, de forma que no es descartable que el plegamiento del pre-rRNA esté de algún modo escondiendo o enmascarando el SRL. Además, los resultados recogidos en el apartado C2 parecen indicar que las ribotoxinas pueden inactivar partículas pre-60S aún no completamente ensambladas, y no sólo ribosomas maduros. Esto podría contribuir a la alta eficiencia de estas toxinas, ya que atacando una ruta celular tan valiosa para la célula estarían potenciando su acción tóxica y probablemente acelerando la muerte celular.

Puesto que la α-sarcina parece actuar también a nivel de la maduración ribosomal, resulta interesante estudiar las consecuencias que tendría la inactivación de partículas pre-60S sobre el conjunto de eventos que comprende la biogénesis de los ribosomas. La falta de subunidades 60S funcionales en el citoplasma, no obstante, no parece provocar ningún desequilibrio en el transporte de subunidades pre-60S o pre-40S a través del poro nuclear (Figura C2.5). Tampoco parece afectar al transporte núcleo-citoplasma de los factores de ensamblaje (Figura C2.3). Uno de los factores analizados es Tif6 (eIF6 en humanos), que se encarga de prevenir la asociación de las dos subunidades del ribosoma cuando éstas no están aún listas para comenzar la traducción (Menne et al. 2007b). Se trataría de un mecanismo de comprobación por el

cual se detectaría que la subunidad está correctamente ensamblada, desencadenando entonces la activación de la GTPasa Efl1 que, en combinación con Sdo1, induciría la liberación de Tif6 de la subunidad 60S, permitiendo la entrada en traducción de esta última. La resolución de la estructura de la subunidad 60S asociada al factor eIF6 (Klinge et al. 2011) muestra cómo este factor interacciona con uL23 y, curiosamente, está colocado muy cerca del SRL, aunque no lo suficientemente como para cubrirlo por completo (Figura D3). En ese sentido, es posible que la α-sarcina pueda tener acceso al SRL incluso en presencia de Tif6 en la subunidad pre-60S, lo que concuerda con los resultados de esta Memoria, en los que la toxina es capaz de cortar partículas pre-60S que contienen Tif6 (Arx1-TAP, Kre35-TAP, Figura D.4). El trabajo de Klinge y colaboradores muestra cómo la proteína uL23 se localiza muy próxima al SRL, por lo que tampoco es descartable que la α-sarcina pueda establecer algún tipo de interacción con ésta en su búsqueda del SRL. Asimismo, las similitudes estructurales y funcionales entre Efl1 y el factor de elongación 2 (EF2) (de la Cruz et al. 2015), presumen un efecto similar de las ribotoxinas sobre la acción del primero tal y como se ha descrito previamente para los factores de elongación EF-Tu y EFG procariotas (García-Ortega et al. 2010). Algo muy interesante de abordar en un futuro especialmente en el contexto de ribosomopatías como el síndrome Shwachman-Diamond donde este paso de la biogénesis ribosomal no se da correctamente (Menne et al. 2007a).

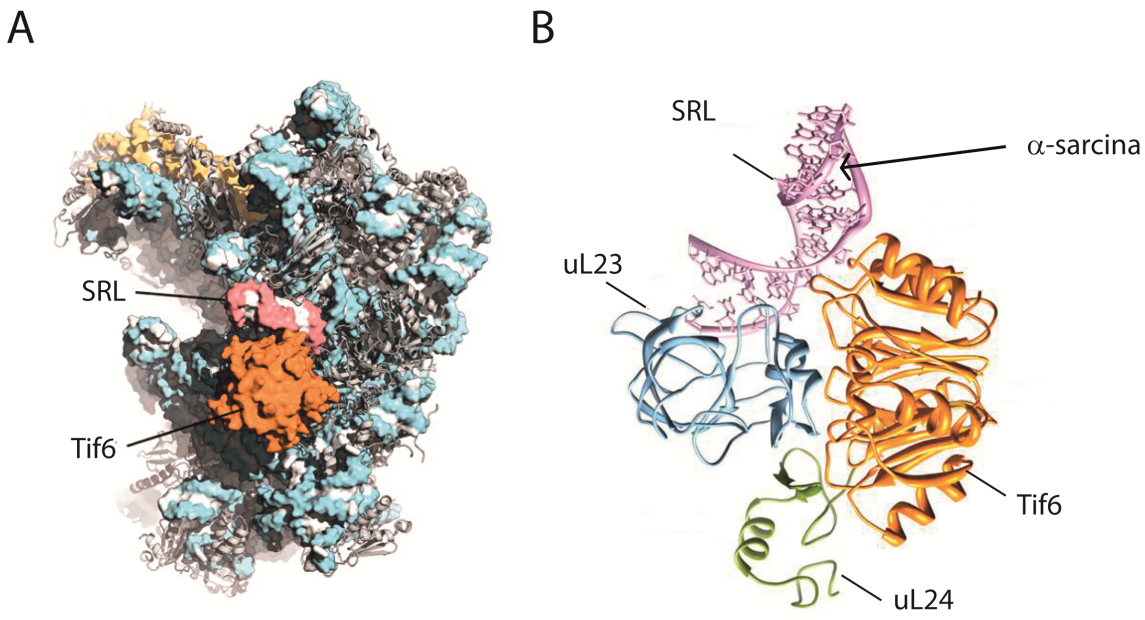


Figura D.3.- Estructura tridimensional de la subunidad mayor del ribosoma unida al factor de ensamblaje Tif6. A) Subunidad 60S de *Tetrahymena termophila*, destacando el SRL y el factor Tif6 (adaptada de Klinge et al. 2011). B) sitio de unión de Tif6 a la subunidad 60S de *S. cerevisiae* (adaptada de Gartmann et al. 2010). Tif6 estaría interaccionando con la proteína uL23 y el SRL. El sitio de corte por la α-sarcina también aparece señalado.

La integridad del SRL es esencial para la correcta interacción con los factores de elongación de la traducción. De hecho, se ha propuesto su participación en la activación de la actividad GTPasa de estos factores, ya que en bacterias el grupo fosfato de la A_{2662} (justo el enlace que corta la α-sarcina) parece contactar con la His84 de EF-Tu y posicionarla en el centro activo para que pueda actuar como base general durante la hidrólisis del GTP (Voorhees et al. 2010, Koch et al. 2015). No obstante, el papel que juega el SRL en la activación de la hidrólisis del GTP no está bien definido y genera controversia. Por ejemplo, la síntesis de péptidos en condiciones independientes de EF-G, no se ve afectada por el corte o despurinación del SRL (Chan and Wool 2008, García-Ortega et al. 2010). Si bien este tipo de traducción no es común, estos resultados se ven apoyados por otros donde se observa que la integridad del SRL no es necesaria para la hidrólisis de GTP por EF-Tu o EF-G (Shi et al. 2012), y el corte por α-sarcina afecta de manera diferente a la unión de EF-Tu y EF-G al ribosoma (García-Ortega et al. 2010). Desde la perspectiva de la maduración ribosomal, se ha propuesto que la interacción de la partícula pre-60S con el factor Efl1 sirva como mecanismo de control del correcto ensamblaje del sitio P y del correcto plegamiento del SRL (Bussiere et al. 2012). Sólo si la subunidad 60S está correctamente ensamblada se liberaría Tif6 y se completaría la maduración de la partícula. Los datos publicados hasta ahora describen el control del sitio P por parte de Efl1 y Sdo1, sin embargo poco o nada se sabe sobre el chequeo del SRL. En los resultados C2 de esta Memoria se muestra como la α-sarcina es capaz de cortar partículas pre-60S que contienen Tif6. Sería interesante evaluar la capacidad de unión y la actividad GTPasa de Efl1 a partículas pre-60S tratadas con sarcina. Si el corte del SRL impide la unión de Efl1 o bloquea la liberación de Tif6, se podría afirmar que la integridad de este bucle es necesaria para la correcta maduración del ribosoma.

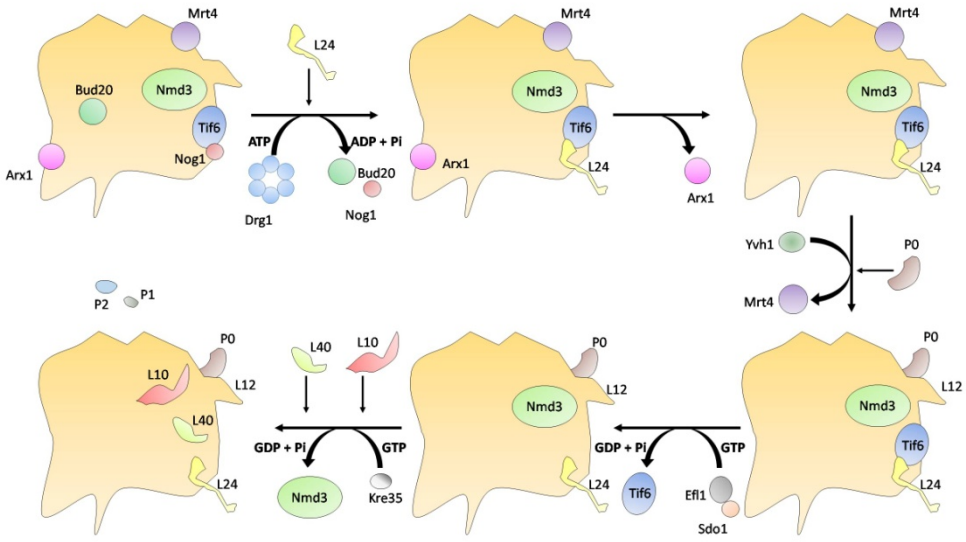


Figura D.4.- Esquema de las etapas de maduración de la subunidad 60S en el citoplasma (simplificada de de la Cruz et al. 2015).

De los resultados presentados en esta Tesis, también se establece que el corte del SRL por parte de la α-sarcina no afecta al procesamiento del rRNA 18S de la subunidad 40S (Figura C2.4). En general, el único efecto que parece tener la acción de la α-sarcina *in vivo* sobre la biogénesis del ribosoma es un aumento exacerbado de la transcripción del pre-rRNA 35S (Figura C2.4). Si bien, esto puede ser una consecuencia directa de la falta de ribosomas funcionales debido al corte del SRL en las subunidades 60S. La célula detectaría una disminución de la traducción, activando la transcripción masiva de rRNA con el objetivo de sintetizar más ribosomas para compensarlo. No obstante, como la velocidad de traducción es menor debido a la acción directa de la ribotoxina sobre los ribosomas maduros, habrá menos proteínas ribosomales disponibles, siendo imposible para la célula aumentar la velocidad de síntesis de nuevos ribosomas. El hecho de que no se puedan sintetizar tampoco los factores proteicos implicados en la biogénesis ribosomal explica también que no se observe una acumulación de éstos en el citoplasma (Figura C2.3). Esto, provoca que el corte del SRL por parte de las ribotoxinas sea letal para las células, que no son capaces de dividirse. La RIP ricina parece presentar un comportamiento semejante al de la α-sarcina en lo que respecta a la biogénesis del ribosoma (Dr. Tchorzewski, comunicación personal), actuando mayoritariamente sobre las últimas etapas de maduración. Esto podría indicar que la biogénesis del ribosoma no es la principal diana de actuación de estas toxinas, sino más bien un efecto colateral de su elevada especificidad. En cualquier caso, parece seguro afirmar que la inactivación de pre-60S citoplásmicas no hace sino potenciar la citotoxicidad de la ricina y la α-sarcina.

Consideraciones finales

Se han tratado distintos aspectos de las ribotoxinas fúngicas, que van desde su función en el contexto biológico del hongo que las produce hasta sus posibles aplicaciones biotecnológicas. Se ha profundizado en el estudio de las relaciones estructura-función de las proteínas α-sarcina y HtA, dos miembros representativos de las ribotoxinas. En concreto, se han asignados nuevas funcionalidades a residuos de los bucles 2 y 3 de la α-sarcina, y estudiado el papel de la horquilla β amino terminal de la HtA en su actividad citotóxica. Además, se ha descrito por primera vez la existencia de una ribotoxina en el hongo entomopatógeno *Metarhizium anisopliae*, de gran interés dado su potencial biológico en el control del mosquito transmisor de la malaria. Esta ribotoxina se ha caracterizado desde un punto de vista estructural y funcional. La comparación del comportamiento de las ribotoxinas α-sarcina y HtA frente a insectos apoya la idea de que en la naturaleza los hongos utilizan estas toxinas como insecticidas, ya sea como defensa frente a depredadores o como una estrategia parasitaria. En conjunto, todo parece apuntar a que las ribotoxinas podrían ser efectivas como biopesticidas en el control biológico de plagas. Si bien, el uso de éstas como herramientas biológicas requiere del conocimiento exhaustivo de su mecanismo de acción. Por ello, en esta Tesis también se ha profundizado en este aspecto,

evidenciándose cómo la α-sarcina parece seguir un mecanismo de interacción con el ribosoma eucariota distinto del que utilizan algunas RIPs como la ricina. Además, se ha demostrado por primera vez cómo las ribotoxinas no actúan sólo sobre ribosomas maduros, sino que también podrían cortar el SRL de partículas pre-60S en las últimas etapas de maduración del ribosoma, potenciando su acción tóxica. Estas observaciones abren la puerta al empleo de las ribotoxinas como herramientas biotecnológicas a la hora de estudiar ciertas enfermedades raras producidas por un mal ensamblaje o funcionamiento del ribosoma.

Conclusiones/Conclusions

- La triada de lisinas K111, K112 y K114 del bucle 3 de la α-sarcina es esencial para el reconocimiento y corte del SRL. Los residuos K111 y K114 parecen,además, estar implicados en la interacción con vesículas lipídicas. Asimismo, se ha puesto de manifiesto una red de interacciones que parece ser esencial para la catálisis y que se establece entre la K114 y la Y48 del centro activo.

- El residuo H82 no parece estar implicado en la actividad ribonucleolítica de la α-sarcina, ni en su interacción con vesículas lipídicas. Pero, si se reemplaza por glutamina (H82Q), tiene lugar un cambio conformacional que expondría residuos susceptibles de interaccionar con lípidos.

- La deleción del bucle 2 completo de la α-sarcina, en el que se encuentra la H82, da lugar a la pérdida de actividad catalítica de la toxina frente a todos los sustratos ensayados. Por lo tanto debe estar involucrado en interacciones esenciales con el centro activo. Sin embargo, no afecta a las interacciones con lípidos.

- Los mutantes de la HtA K115E, K118E, K123E en el bucle 5, y Δ(8-15) en la horquilla β amino-terminal, mantienen su actividad ribonucleolítica específica, aunque ésta es menor que la de la forma silvestre. Estos resultados apoyan la hipótesis de una compensación entre ambas regiones de la proteína y una mejor adaptabilidad del centro activo de la HtA con respecto al de la α-sarcina.

- La horquilla β amino terminal de la HtA y el residuo K123 del bucle 5 parecen jugar un papel importante en la actividad insecticida de esta proteína sobre células de insecto en cultivo.

- Se ha aislado y caracterizado una nueva ribotoxina, denominada anisoplina (Anp), producida por el hongo entomopatógeno *Metarhizium anisopliae,* que posee una estructura muy similar a la de la HtA y presenta la actividad ribonucleolítica específica típica de las ribotoxinas.

- La HtA no es la única ribotoxina con propiedades insecticidas. La α-sarcina es también capaz de actuar sobre larvas, células y ribosomas de insectos con la misma actividad que la HtA. La Anp también posee propiedades insecticidas frente a células cultivadas *in vitro*.

- La α-sarcina no interacciona con las proteínas ácidas del tallo ribosómico eucariota para acceder y cortar el SRL, por lo que el mecanismo de reconocimiento del SRL parece ser diferente al que utilizan las RIPs del tipo de la ricina.

- Una expresión basal de la α-sarcina en *S. cerevisiae* resulta letal para todas las cepas ensayadas. Por eso el sistema de expresión elegido para producir esta proteína intracelularmente en la levadura resulta de vital importancia. En este sentido, los mejores resultados se han obtenido al utilizar el sistema de expresión de α-sarcina en levaduras bajo control del promotor de galactosa.

- La α-sarcina es capaz no sólo de cortar específicamente ribosomas maduros, sino también partículas pre-60S en las últimas etapas de la maduración ribosomal, principalmente en el citoplasma.

- El pre-rRNA 27S no es cortado por la α-sarcina a pesar de que ésta puede acceder al núcleo de la célula donde se localiza.

- La actividad ribonucleolítica de la α-sarcina no afecta a procesos de la ruta de biogénesis de ribosoma como el transporte de las subunidades pre-60S y pre-40S o el reciclaje de los factores que contribuyen a su correcta maduración. No obstante, parece desencadenar una señal que aumenta la transcripción de pre-rRNA 35S para compensar la falta de ribosomas funcionales en el citoplasma.

- The triad of lysines K111, K112 and K114 of loop 3 of α-sarcin is essential to recognize and cleave the SRL. Moreover, residues K111 and K114 seem to be involved in approaching lipid vesicles. Also, the interaction between residues K114 and Y48 seems to be essential for catalysis.

- Residue H82 does not seem to be involved in the ribonucleolytic activity of α-sarcin, nor in its interaction with lipid vesicles. However, when this residue is replaced by glutamine (H82Q), a conformational change occurs that would expose other residues capable of interacting with lipids.

- Deletion of the entire loop 2 of α-sarcin, which contains H82, results in the loss of catalytic activity of the toxin against all substrates assayed. Therefore, it must be involved in essential interactions with the active site. However, deletion of this loop does not affect its ability to interactwith lipids.

- HtA mutant variants K115E, K118E, K123E of loop 5 and Δ(8-15) of the N-terminal β-hairpin retain their specific ribonucleolytic activity, but it is significantly lower than the observed for wild type HtA. These results support the hypothesis that loop 5 compensates the differences found in the N-terminal β-hairpin of HtA when compared to α-sarcin, and that the active site of HtA seems more adaptable than that of α-sarcin.

- The N-terminal β-hairpin of HtA and the residue K123 of loop 5 seem to participate in the insecticidal activity of the protein on insect cell cultures.

- A new ribotoxin, named anisoplin (Anp),which is produced by the entomopathogenic fungus *Metarhizium anisopliae,* has been characterized, showing a similar structure than HtA and the characteristicspecificribonucleolytic activity of ribotoxins.

- HtA is not the only ribotoxin with insecticidal properties. α-sarcin is also capable of acting on insect larvae, cells and ribosomes with the same activity as HtA. Anp also displays these insecticidal properties against insect cell cultures.

-α-Sarcin does not interact with the acidic proteins of the eukaryotic ribosomal stalk to access and cleave the SRL. Therefore, the mechanism of recognizing the SRL is quite different from that one used by RIPs like ricin.

- Intracellular basal expression of α-sarcin in *S.cerevisiae* is lethal to all strains used. That is why the expression system chosen for its intracellular production in yeast is of

great importance. In this regard, best results have been obtained with the α-sarcin expression system based on galactose promoter.

- α-sarcin is capable of inactivating not only mature ribosomes, but also late pre-60S particles, mainly during cytoplasmic maturation.

- 27S pre-rRNA is not cleaved by α-sarcin even though the toxin can access the nucleus, where it is located.

- The ribonucleolytic activity of α-sarcin does not affect processes of the pathway of ribosome biogenesis like pre-60S and pre-40S subunit transport or the recycling of trans-acting factors involved in the correct assembly of ribosomes. However, α-sarcin activity seems to trigger a signal that enhances 35S pre-rRNA transcription to compensate the lack of functional ribosomes in the cytoplasm.

Bibliografía/References

Alford, S. C., Pearson, J. D., Carette, A., Ingham, R. J. and Howard, P. L. (2009). "α-Sarcin catalytic activity is not required for cytotoxicity." *BMC Biochem* **10** (1): 9.

Altvater, M., Chang, Y., Melnik, A., Occhipinti, L., Schutz, S., Rothenbusch, U., Picotti, P. and Panse, V. G. (2012). "Targeted proteomics reveals compositional dynamics of 60S pre-ribosomes after nuclear export." *Mol Syst Biol* **8**: 628.

Álvarez-García, E., García-Ortega, L., Verdun, Y., Bruix, M., Martínez-del-Pozo, A. and Gavilanes, J. G. (2006). "Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of α-sarcin." *Biol Chem* **387** (5): 535-541.

Álvarez-García, E., Alegre-Cebollada, J., Batanero, E., Monedero, V., Pérez-Martínez, G., García-Fernández, R., Gavilanes, J. G. and del Pozo, A. M. (2008). "*Lactococcus lactis* as a vehicle for the heterologous expression of fungal ribotoxin variants with reduced IgE-binding affinity." *J Biotechnol* **134** (1-2): 1-8.

Álvarez-García, E., Martínez-del-Pozo, A. and Gavilanes, J. G. (2009). "Role of the basic character of α-sarcin's NH_2-terminal β-hairpin in ribosome recognition and phospholipid interaction." *Arch Biochem Biophys* **481** (1): 37-44.

Álvarez-García, E., Batanero, E., García-Fernández, R., Villalba, M., Gavilanes, J. G. and Martínez-del-Pozo, A. (2010). "A deletion variant of the *Aspergillus fumigatus* ribotoxin Asp f 1 induces an attenuated airway inflammatory response in a mouse model of sensitization." *J Investig Allergol Clin Immunol* **20** (1): 69-75.

Arruda, L. K., Mann, B. J. and Chapman, M. D. (1992). "Selective expression of a major allergen and cytotoxin, Asp f I, in *Aspergillus fumigatus*. Implications for the immunopathogenesis of Aspergillus-related diseases." *J Immunol* **149** (10): 3354-3359.

Ayub, M. J., Smulski, C. R., Ma, K. W., Levin, M. J., Shaw, P. C. and Wong, K. B. (2008). "The C-terminal end of P proteins mediates ribosome inactivation by trichosanthin but does not affect the pokeweed antiviral protein activity." *Biochem Biophys Res Commun* **369** (2): 314-319.

Bautista-Santos, A. and Zinker, S. (2014). "The P1/P2 protein heterodimers assemble to the ribosomal stalk at the moment when the ribosome is committed to translation but not to the native 60S ribosomal subunit in Saccharomyces cerevisiae." *Biochemistry* **53** (25): 4105-4112.

Benton, B. M., Eng, W. K., Dunn, J. J., Studier, F. W., Sternglanz, R. and Fisher, P. A. (1990). "Signal-mediated import of bacteriophage T7 RNA polymerase into the Saccharomyces cerevisiae nucleus and specific transcription of target genes." *Molecular and Cellular Biology* **10** (1): 353-360.

Berenbaum, M. R. and Eisner, T. (2008). "Ecology. Bugs' bugs." *Science* **322** (5898): 52-53.

Brandhorst, T. and Kenealy, W. R. (1995). "Effects of leader sequences upon the heterologous expression of restrictocin in *Aspergillus nidulans* and *Aspergillus niger*." *Can J Microbiol* **41** (7): 601-611.

Brandhorst, T., Dowd, P. F. and Kenealy, W. R. (1996). "The ribosome-inactivating protein restrictocin deters insect feeding on *Aspergillus restrictus*." *Microbiology* **142 (Pt 6)**: 1551-1556.

Brandhorst, T., Dowd, P. F. and Kenealy, W. R. (2001). "The effect of fungal ribosome inactivating proteins upon feeding choice in *C. freemani*, and indications of a mutualistic relationship with *A. restrictus*. Environmental mycology." *Mycopathologia* **152** (3): 155-158.

Brandhorst, T. T. and Kenealy, W. R. (1992). "Production and localization of restrictocin in *Aspergillus restrictus*." *J. Gen. Microbiol.* **138**: 1429-1435.

Brina, D., Miluzio, A., Ricciardi, S. and Biffo, S. (2014). "eIF6 anti-association activity is required for ribosome biogenesis, translational control and tumor progression." *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*.

Bussiere, C., Hashem, Y., Arora, S., Frank, J. and Johnson, A. W. (2012). "Integrity of the P-site is probed during maturation of the 60S ribosomal subunit." *J Cell Biol* **197** (6): 747-759.

Carlini, C. R. and Grossi-de-Sa, M. F. (2002). "Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides." *Toxicon* **40** (11): 1515-1539.

Carreras-Sangrà, N., Tomé-Amat, J., García-Ortega, L., Batt, C. A., Oñaderra, M., Martínez-del-Pozo, A., Gavilanes, J. G. and Lacadena, J. (2012). "Production and characterization of a colon cancer-specific immunotoxin based on the fungal ribotoxin α-sarcin." *Protein Eng Des Sel*.

Casselton, L. and Zolan, M. (2002). "The art and design of genetic screens: filamentous fungi." *Nat Rev Genet* **3** (9): 683-697.

Castaño-Rodríguez, C., Olombrada, M., Partida-Hanon, A., Lacadena, J., Oñaderra, M., Gavilanes, J. G., García-Ortega, L. and Martínez-del-Pozo, A. (2015). "Involvement of loops 2 and 3 of α-sarcin on its ribotoxic activity." *Toxicon* **96C**: 1-9.

Chan, Y. L. and Wool, I. G. (2008). "The Integrity of the Sarcin/Ricin Domain of 23 S Ribosomal RNA Is Not Required for Elongation Factor-independent Peptide Synthesis." *J Mol Biol*.

Chen, H. (2012). "Exploiting the Intron-splicing Mechanism of Insect Cells to Produce Viral Vectors Harboring Toxic Genes for Suicide Gene Therapy." *Mol Ther Nucleic Acids* **1**: e57.

Chiou, J. C., Li, X. P., Remacha, M., Ballesta, J. P. and Tumer, N. E. (2008). "The ribosomal stalk is required for ribosome binding, depurination of the rRNA and cytotoxicity of ricin A chain in *Saccharomyces cerevisiae*." *Mol Microbiol* **70** (6): 1441-1452.

Coca, M., Bortolotti, C., Rufat, M., Penas, G., Eritja, R., Tharreau, D., Martínez del Pozo, A., Messeguer, J. and San Segundo, B. (2004). "Transgenic Rice Plants Expressing the Antifungal AFP Protein from *Aspergillus Giganteus* Show Enhanced Resistance to the Rice Blast Fungus *Magnaporthe Grisea*." *Plant Mol Biol* **54** (2): 245-259.

Cory, J. S., Hirst, M. L., Williams, T., Hails, R. S., Goulson, D., Green, B. M., Carty, T. M., Possee, R. D., Cayley, P. J. and Bishop, D. H. L. (1994). "Field trial of a genetically improved baculovirus insecticide." *Nature* **370** (6485): 138-140.

de Bekker, C., Smith, P. B., Patterson, A. D. and Hughes, D. P. (2013). "Metabolomics reveals the heterogeneous secretome of two entomopathogenic fungi to ex vivo cultured insect tissues." *PLoS One* **8** (8): e70609.

de la Cruz, J., Karbstein, K. and Woolford, J. L., Jr. (2015). "Functions of ribosomal proteins in assembly of eukaryotic ribosomes in vivo." *Annu Rev Biochem* **84**: 93-129.

Diaconu, M., Kothe, U., Schlunzen, F., Fischer, N., Harms, J. M., Tonevitsky, A. G., Stark, H., Rodnina, M. V. and Wahl, M. C. (2005). "Structural basis for the function of the ribosomal L7/12 stalk in factor binding and GTPase activation." *Cell* **121** (7): 991-1004.

Endo, Y., Chan, Y.-L., Lin, A., Tsurugi, K. and Wool, I. (1988). "The cytotoxins α-sarcin and ricin retain their specificity when tested on a synthetic oligoribonucleotide (35-mer) that mimics a region of 28 S ribosomal ribonucleic acid." *J Biol Chem* **263** (17): 7917-7920.

Endo, Y., Tsurugi, K., Oka, T. and Natori, Y. (1993a). "The biosynthesis of a cytotoxic protein, alpha-sarcin, in a mold of *Aspergillus giganteus*.I Synthesis of pre- and pro-alpha-sarcin in vitro." *The Tokushima Journal of Experimental Medicine* **40** (1-2): 1-6.

Endo, Y., Oka, T., Yokota, S., Tsurugi, K. and Natori, Y. (1993b). "The biosynthesis of a cytotoxic protein, alpha-sarcin, in a mold of *Aspergillus giganteus*. II. Maturation of precursor form of alpha-sarcin in vivo." *Tokushima J Exp Med* **40** (1-2): 7-12.

Evans, J. D. and Schwarz, R. S. (2011). "Bees brought to their knees: microbes affecting honey bee health." *Trends Microbiol* **19** (12): 614-620.

Faria, M. R. d. and Wraight, S. P. (2007). "Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types." *Biological Control* **43** (3): 237-256.

Faza, M. B., Chang, Y., Occhipinti, L., Kemmler, S. and Panse, V. G. (2012). "Role of Mex67-Mtr2 in the nuclear export of 40S pre-ribosomes." *PLoS Genet* **8** (8): e1002915.

Galagan, J. E., Calvo, S. E., Cuomo, C., Ma, L. J., Wortman, J. R., Batzoglou, S., Lee, S. I., Basturkmen, M., Spevak, C. C., Clutterbuck, J., Kapitonov, V., Jurka, J., Scazzocchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G. H., Draht, O., Busch, S., D'Enfert, C., Bouchier, C., Goldman, G. H., Bell-Pedersen, D., Griffiths-Jones, S., Doonan, J. H., Yu, J., Vienken, K., Pain, A., Freitag, M., Selker, E. U., Archer, D. B., Penalva, M. A., Oakley, B. R., Momany, M., Tanaka, T., Kumagai, T., Asai, K., Machida, M., Nierman, W. C., Denning, D. W., Caddick, M., Hynes, M., Paoletti, M., Fischer, R., Miller, B., Dyer, P., Sachs, M. S., Osmani, S. A. and Birren, B. W. (2005). "Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*." *Nature* **438** (7071): 1105-1115.

García-Mayoral, F., García-Ortega, L., Álvarez-García, E., Bruix, M., Gavilanes, J. G. and Martínez-del-Pozo, A. (2005). "Modeling the highly specific ribotoxin recognition of ribosomes." *FEBS Lett* **579** (30): 6859-6864.

Garcia-Ortega, L., Lacadena, J., Lacadena, V., Masip, M., De Antonio, C., Martinez-Ruiz, A. and Martinez Del Pozo, A. (2000). "The solubility of the ribotoxin alpha-sarcin, produced as a recombinant protein in *Escherichia coli*, is increased in the presence of thioredoxin." *Lett Appl Microbiol* **30** (4): 298-302.

García-Ortega, L., Lacadena, J., Mancheño, J. M., Oñaderra, M., Kao, R., Davies, J., Olmo, N., Martínez-del-Pozo, A. and Gavilanes, J. G. (2001). "Involvement of the amino-terminal β-hairpin of the *Aspergillus* ribotoxins on the interaction with membranes and nonspecific ribonuclease activity." *Protein Sci* **10** (8): 1658-1668.

García-Ortega, L., Masip, M., Mancheño, J. M., Oñaderra, M., Lizarbe, M. A., García-Mayoral, M. F., Bruix, M., Martínez-del-Pozo, A. and Gavilanes, J. G. (2002). "Deletion of the NH_2-terminal β-hairpin of the ribotoxin α-sarcin produces a nontoxic but active ribonuclease." *J Biol Chem* **277** (21): 18632-18639.

García-Ortega, L., Lacadena, J., Villalba, M., Rodríguez, R., Crespo, J. F., Rodríguez, J., Pascual, C., Olmo, N., Oñaderra, M., Martínez-del-Pozo, A. and Gavilanes, J. G. (2005). "Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Asp f 1 displaying reduced IgE binding." *FEBS J* **272** (10): 2536-2544.

García-Ortega, L., Alvarez-Garcia, E., Gavilanes, J. G., Martinez-del-Pozo, A. and Joseph, S. (2010). "Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding." *Nucleic Acids Res* **38** (12): 4108-4119.

Gartmann, M., Blau, M., Armache, J. P., Mielke, T., Topf, M. and Beckmann, R. (2010). "Mechanism of eIF6-mediated inhibition of ribosomal subunit joining." *J Biol Chem* **285** (20): 14848-14851.

Gasset, M., Martínez-del-Pozo, A., Oñaderra, M. and Gavilanes, J. G. (1989). "Study of the interaction between the antitumour protein α-sarcin and phospholipid vesicles." *Biochem J* **258** (2): 569-575.

Gasset, M., Oñaderra, M., Thomas, P. G. and Gavilanes, J. G. (1990). "Fusion of phospholipid vesicles produced by the anti-tumour protein α-sarcin." *Biochem J* **265** (3): 815-822.

Gerhardy, S., Menet, A., Peña, C., Petkowski, J. and Panse, V. (2014). "Assembly and nuclear export of pre-ribosomal particles in budding yeast." *Chromosoma* **123** (4): 327-344.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Kohl, J., Marrone, P., Morin, L. and Stewart, A. (2012). "Have biopesticides come of age?" *Trends Biotechnol* **30** (5): 250-258.

Gonzalo, P. and Reboud, J. P. (2003). "The puzzling lateral flexible stalk of the ribosome." *Biol Cell* **95** (3-4): 179-193.

Gramkow, A. W., Perecmanis, S., Sousa, R. L., Noronha, E. F., Felix, C. R., Nagata, T. and Ribeiro, B. M. (2010). "Insecticidal activity of two proteases against *Spodoptera frugiperda* larvae infected with recombinant baculoviruses." *Virol J* **7**: 143.

Grela, P., Li, X. P., Tchorzewski, M. and Tumer, N. E. (2014). "Functional divergence between the two P1-P2 stalk dimers on the ribosome in their interaction with ricin A chain." *Biochem J* **460** (1): 59-67.

Guarinos, E., Remacha, M. and Ballesta, J. P. (2001). "Asymmetric interactions between the acidic P1 and P2 proteins in the *Saccharomyces cerevisiae* ribosomal stalk." *J Biol Chem* **276** (35): 32474-32479.

Herrero-Galán, E., Lacadena, J., Martínez-del-Pozo, A., Boucias, D. G., Olmo, N., Oñaderra, M. and Gavilanes, J. G. (2008). "The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin." *Proteins* **72** (1): 217-228.

Herrero-Galán, E., García-Ortega, L., Lacadena, J., Martínez-Del-Pozo, A., Olmo, N., Gavilanes, J. G. and Oñaderra, M. (2012a). "Implication of an Asp residue in the ribonucleolytic activity of hirsutellin A reveals new electrostatic interactions at the active site of ribotoxins." *Biochimie* **94** (2): 427-433.

Herrero-Galán, E., García-Ortega, L., Lacadena, J., Martínez-Del-Pozo, A., Olmo, N., Gavilanes, J. G. and Oñaderra, M. (2012b). "A non-cytotoxic but ribonucleolytically specific ribotoxin variant: implication of tryptophan residues in the cytotoxicity of hirsutellin A." *Biol Chem* **393** (6): 449-456.

Hudak, K. A., Dinman, J. D. and Tumer, N. E. (1999). "Pokeweed antiviral protein accesses ribosomes by binding to L3." *J Biol Chem* **274** (6): 3859-3864.

Inceoglu, A. B., Kamita, S. G., Hinton, A. C., Huang, Q., Severson, T. F., Kang, K. and Hammock, B. D. (2001). "Recombinant baculoviruses for insect control." *Pest Manag Sci* **57** (10): 981-987.

Kanga, L. H., James, R. R. and Boucias, D. G. (2002). "*Hirsutella thompsonii* and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor,* a honey bee parasite." *J Invertebr Pathol* **81** (3): 175-184.

Kemmler, S., Occhipinti, L., Veisu, M. and Panse, V. G. (2009). "Yvh1 is required for a late maturation step in the 60S biogenesis pathway." *The Journal of Cell Biology* **186** (6): 863-880.

Kim, C. S., Lee, J. B., Kim, B. S., Nam, Y. H., Shin, K. S., Kim, J. W., Kim, J. E. and Kwon, G. S. (2014). "A technique for the prevention of greenhouse whitefly (*Trialeurodes vaporariorum*) using the entomopathogenic fungus *Beauveria bassiana* M130." *J Microbiol Biotechnol* **24** (1): 1-7.

Klinge, S., Voigts-Hoffmann, F., Leibundgut, M., Arpagaus, S. and Ban, N. (2011). "Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6." *Science* **334** (6058): 941-948.

Koch, M., Flur, S., Kreutz, C., Ennifar, E., Micura, R. and Polacek, N. (2015). "Role of a ribosomal RNA phosphate oxygen during the EF-G-triggered GTP hydrolysis." *Proc Natl Acad Sci U S A* **112** (20): E2561-2568.

Korennykh, A. V., Piccirilli, J. A. and Correll, C. C. (2006). "The electrostatic character of the ribosomal surface enables extraordinarily rapid target location by ribotoxins." *Nat Struct Mol Biol*.

Korennykh, A. V., Correll, C. C. and Piccirilli, J. A. (2007). "Evidence for the importance of electrostatics in the function of two distinct families of ribosome inactivating toxins." *RNA* **13** (9): 1391-1396.

Kreitman, R. J. (2001). "Toxin-labeled monoclonal antibodies." *Curr Pharm Biotechnol* **2** (4): 313-325.

Kreitman, R. J. (2006). "Immunotoxins for targeted cancer therapy." *Aaps J* **8** (3): E532-551.

Kurup, V. P., Banerjee, B., Murali, P. S., Greenberger, P. A., Krishnan, M., Hari, V. and Fink, J. N. (1998). "Immunodominant peptide epitopes of allergen, Asp f 1 from the fungus *Aspergillus fumigatus*." *Peptides* **19** (9): 1469-1477.

Lacadena, J., Mancheño, J. M., Martínez-Ruiz, A., Martínez-del-Pozo, A., Gasset, M., Oñaderra, M. and Gavilanes, J. G. (1995). "Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin." *Biochem J* **309**: 581-586.

Lacadena, J., Martínez-del-Pozo, A., Lacadena, V., Martínez-Ruiz, A., Mancheño, J. M., Oñaderra, M. and Gavilanes, J. G. (1998). "The cytotoxin α-sarcin behaves as a cyclizing ribonuclease." *FEBS Lett* **424** (1-2): 46-48.

Lacadena, J., Martínez-del-Pozo, A., Martínez-Ruiz, A., Pérez-Cañadillas, J. M., Bruix, M., Mancheño, J. M., Oñaderra, M. and Gavilanes, J. G. (1999). "Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin α-sarcin." *Proteins* **37** (3): 474-484.

Lacadena, J., Álvarez-García, E., Carreras-Sangrà, N., Herrero-Galán, E., Alegre-Cebollada, J., García-Ortega, L., Oñaderra, M., Gavilanes, J. G. and Martínez-del-Pozo, A. (2007). "Fungal ribotoxins: molecular dissection of a family of natural killers." *FEMS Microbiol Rev* **31**: 212-237.

Lafontaine, D. L. J. (2015). "Noncoding RNAs in eukaryotic ribosome biogenesis and function." *Nat Struct Mol Biol* **22** (1): 11-19.

Lamy, B. and Davies, J. (1991). "Isolation and nucleotide sequence of the *Aspergillus restrictus* gene coding for the ribonucleolytic toxin restrictocin and its expression in *Aspergillus nidulans*: the leader sequence protects producing strains from suicide." *Nucleic Acids Res* **19** (5): 1001-1006.

Lapadula, W. J., Sanchez-Puerta, M. V. and Ayub, M. J. (2012). "Convergent evolution led ribosome inactivating proteins to interact with ribosomal stalk." *Toxicon* **59** (3): 427-432.

Li, X. P., Chiou, J. C., Remacha, M., Ballesta, J. P. and Tumer, N. E. (2009). "A two-step binding model proposed for the electrostatic interactions of ricin a chain with ribosomes." *Biochemistry* **48** (18): 3853-3863.

Li, X. P., Kahn, P. C., Kahn, J. N., Grela, P. and Tumer, N. E. (2013). "Arginine residues on the opposite side of the active site stimulate the catalysis of ribosome depurination by ricin A chain by interacting with the P-protein stalk." *J Biol Chem* **288** (42): 30270-30284.

Liu, J. C., Boucias, D. G., Pendland, J. C., Liu, W. Z. and Maruniak, J. (1996). "The mode of action of hirsutellin A on eukaryotic cells." *J Invertebr Pathol* **67** (3): 224-228.

Liu, W.-Z., Boucias, D. G. and McCoy, C. W. (1995). "Extraction and characterization of the insecticidal toxin hirsutellin A produced by *Hirsutella thompsonii* var. *thompsonii*." *Experimental Mycology* **19**: 254-262.

Lo, K.-Y., Li, Z., Wang, F., Marcotte, E. M. and Johnson, A. W. (2009). "Ribosome stalk assembly requires the dual-specificity phosphatase Yvh1 for the exchange of Mrt4 with P0." *The Journal of Cell Biology* **186** (6): 849-862.

Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., Abe, K., Gomi, K., Horiuchi, H., Kitamoto, K., Kobayashi, T., Takeuchi, M., Denning, D. W., Galagan, J. E., Nierman, W. C., Yu, J., Archer, D. B., Bennett, J. W., Bhatnagar, D., Cleveland, T. E., Fedorova, N. D., Gotoh, O., Horikawa, H., Hosoyama, A., Ichinomiya, M., Igarashi, R., Iwashita, K., Juvvadi, P. R., Kato, M., Kato, Y., Kin, T., Kokubun, A., Maeda, H., Maeyama, N., Maruyama, J., Nagasaki, H., Nakajima, T., Oda, K., Okada, K., Paulsen, I., Sakamoto, K., Sawano, T., Takahashi, M., Takase, K., Terabayashi, Y., Wortman, J. R., Yamada, O., Yamagata, Y., Anazawa, H., Hata, Y., Koide, Y., Komori, T., Koyama, Y., Minetoki, T., Suharnan, S., Tanaka, A., Isono, K., Kuhara, S., Ogasawara, N. and Kikuchi, H. (2005). "Genome sequencing and analysis of *Aspergillus oryzae*." *Nature* **438** (7071): 1157-1161.

Maimala, S., Tartar, A., Boucias, D. and Chandrapatya, A. (2002). "Detection of the toxin Hirsutellin A from *Hirsutella thompsonii*." *J Invertebr Pathol* **80** (2): 112-126.

Mancheño, J. M., Gasset, M., Albar, J. P., Lacadena, J., Martinez-del-Pozo, A., Oñaderra, M. and Gavilanes, J. G. (1995). "Membrane interaction of a β-structure-forming synthetic peptide comprising the 116-139th sequence region of the cytotoxic protein α-sarcin." *Biophys J* **68** (6): 2387-2395.

Mancheño, J. M., Martínez-del-Pozo, A., Albar, J. P., Oñaderra, M. and Gavilanes, J. G. (1998). "A peptide of nine amino acid residues from α-sarcin cytotoxin is a membrane-perturbing structure." *Journal of Peptide Research* **51** (2): 142-148.

Marheineke, K., Grunewald, S., Christie, W. and Reilander, H. (1998). "Lipid composition of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) insect cells used for baculovirus infection." *FEBS Lett* **441** (1): 49-52.

Martínez-del-Pozo, A., Gasset, M., Oñaderra, M. and Gavilanes, J. G. (1988). "Conformational study of the antitumor protein α-sarcin." *Biochim Biophys Acta* **953** (3): 280-288.

Martinez-Ruiz, A., Martinez del Pozo, A., Lacadena, J., Onaderra, M. and Gavilanes, J. G. (1999). "Hirsutellin A displays significant homology to microbial extracellular ribonucleases." *J Invertebr Pathol* **74** (1): 96-97.

Martínez-Ruiz, A., Martínez-del-Pozo, A., Lacadena, J., Mancheño, J. M., Oñaderra, M., López-Otín, C. and Gavilanes, J. G. (1998). "Secretion of recombinant pro- and mature fungal α-sarcin ribotoxin by the methylotrophic yeast *Pichia pastoris*: The Lys-Arg motif is required for maturation." *Protein Expr Purif* **12**: 315-322.

Martínez-Ruiz, A., Martínez-del-Pozo, A., Lacadena, J., Oñaderra, M. and Gavilanes, J. G. (1999a). "Hirsutellin A displays significant homology to microbial extracellular ribonucleases." *J Invertebr Pathol* **74** (1): 96-97.

Martínez-Ruiz, A., Kao, R., Davies, J. and Martínez-del-Pozo, A. (1999b). "Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed." *Toxicon* **37** (11): 1549-1563.

May, K. L., Li, X. P., Martinez-Azorin, F., Ballesta, J. P., Grela, P., Tchorzewski, M. and Tumer, N. E. (2012). "The P1/P2 proteins of the human ribosomal stalk are required for ribosome binding and depurination by ricin in human cells." *FEBS J* **279** (20): 3925-3936.

May, K. L., Yan, Q. and Tumer, N. E. (2013). "Targeting ricin to the ribosome." *Toxicon* **69**: 143-151.

Mazet, I. and Vey, A. (1995). "Hirsutellin A, a toxic protein produced in vitro by *Hirsutella thompsonii*." *Microbiology* **141**: 1343-1348.

McCann, K. L. and Baserga, S. J. (2013). "Mysterious Ribosomopathies." *Science* **341** (6148): 849-850.

McCluskey, A. J., Poon, G. M., Bolewska-Pedyczak, E., Srikumar, T., Jeram, S. M., Raught, B. and Gariepy, J. (2008). "The catalytic subunit of shiga-like toxin 1 interacts with ribosomal stalk proteins and is inhibited by their conserved C-terminal domain." *J Mol Biol* **378** (2): 375-386.

McCoy, C. W. and Couch, T. L. (1982). "Microbial control of the citrus rust mite with the mycoacaricide, Mycar." *The Florida Entomologist* **65** (1): 116-126.

Menne, T. F., Goyenechea, B., Sanchez-Puig, N., Wong, C. C., Tonkin, L. M., Ancliff, P. J., Brost, R. L., Costanzo, M., Boone, C. and Warren, A. J. (2007a). "The Shwachman-Bodian-Diamond syndrome protein mediates translational activation of ribosomes in yeast." *Nat Genet* **39** (4): 486-495.

Menne, T. F., Goyenechea, B., Sanchez-Puig, N., Wong, C. C., Tonkin, L. M., Ancliff, P. J., Brost, R. L., Costanzo, M., Boone, C. and Warren, A. J. (2007b). "The Shwachman-Bodian-Diamond syndrome protein mediates translational activation of ribosomes in yeast." *Nat Genet* **39** (4): 486-495.

Miller, S. P. and Bodley, J. W. (1988). "The ribosomes of *Aspergillus giganteus* are sensitive to the cytotoxic action of alpha-sarcin." *FEBS Lett* **229** (2): 388-390.

Mohr, D., Wintermeyer, W. and Rodnina, M. V. (2002). "GTPase activation of elongation factors Tu and G on the ribosome." *Biochemistry* **41** (41): 12520-12528.

Moreno, A. B., Penas, G., Rufat, M., Bravo, J. M., Estopa, M., Messeguer, J. and San Segundo, B. (2005). "Pathogen-induced production of the antifungal AFP protein from *Aspergillus giganteus* confers resistance to the blast fungus *Magnaporthe grisea* in transgenic rice." *Mol Plant Microbe Interact* **18** (9): 960-972.

Nakhoul, H., Ke, J., Zhou, X., Liao, W., Zeng, S. X. and Lu, H. (2014). "Ribosomopathies: Mechanisms of Disease." *Clinical Medicine Insights: Blood Disorders* **7**: 7-16.

Narla, A. and Ebert, B. L. (2010). "Ribosomopathies: human disorders of ribosome dysfunction." *Blood* **115** (16): 3196-3205.

Oka, T., Natori, Y., Tanaka, S., Tsurugi, K. and Endo, Y. (1990). "Complete nucleotide sequence of cDNA for the cytotoxin alpha sarcin." *Nucleic Acids Res* **18** (7): 1897.

Olmo, N., Turnay, J., González de Buitrago, G., López de Silanes, I., Gavilanes, J. G. and Lizarbe, M. A. (2001). "Cytotoxic mechanism of the ribotoxin α-sarcin. Induction of cell death via apoptosis." *Eur J Biochem* **268** (7): 2113-2123.

Olombrada, M., Herrero-Galán, E., Tello, D., Oñaderra, M., Gavilanes, J. G., Martinez-del-Pozo, A. and García-Ortega, L. (2013). "Fungal extracellular ribotoxins as insecticidal agents." *Insect Biochem Mol Biol* **43** (1): 39-46.

Olombrada, M., Rodriguez-Mateos, M., Prieto, D., Pla, J., Remacha, M., Martinez-del-Pozo, A., Gavilanes, J. G., Ballesta, J. P. and Garcia-Ortega, L. (2014). "The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by alpha-sarcin of the eukaryotic ribosome." *Biochemistry* **53** (10): 1545-1547.

Olombrada, M., Martínez-del-Pozo, A., Medina, P., Budia, F., Gavilanes, J. G. and García-Ortega, L. (2014a). "Fungal ribotoxins: Natural protein-based weapons against insects." *Toxicon* **83**: 69-74.

Olombrada, M., Rodríguez-Mateos, M., Prieto, D., Pla, J., Remacha, M., Martínez-del-Pozo, A., Gavilanes, J. G., Ballesta, J. P. and García-Ortega, L. (2014b). "The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by α-sarcin of the eukaryotic ribosome." *Biochemistry* **53** (10): 1545-1547.

Olson, B. H. and Goerner, G. L. (1965). "Alpha sarcin, a new antitumor agent. I. Isolation, purification, chemical composition, and the identity of a new amino acid." *Appl Microbiol* **13**: 314-321.

Panse, V. G. and Johnson, A. W. (2010). "Maturation of eukaryotic ribosomes: acquisition of functionality." *Trends in Biochemical Sciences* **35** (5): 260-266.

Peng, C. Y., Zhou, X. and Kaya, H. K. (2002). "Virulence and site of infection of the fungus, *Hirsutella thompsonii*, to the honey bee ectoparasitic mite, *Varroa destructor*." *J Invertebr Pathol* **81** (3): 185-195.

Pérez-Cañadillas, J. M., Santoro, J., Campos-Olivas, R., Lacadena, J., Martínez-del-Pozo, A., Gavilanes, J. G., Rico, M. and Bruix, M. (2000). "The highly refined solution structure of the cytotoxic ribonuclease α-sarcin reveals the structural requirements for substrate recognition and ribonucleolytic activity." *J Mol Biol* **299** (4): 1061-1073.

Perez-Cañadilllas, J. M., Garcia-Mayoral, M. F., Laurents, D. V., Martinez del Pozo, A., Gavilanes, J. G., Rico, M. and Bruix, M. (2003). "Tautomeric state of alpha-sarcin histidines. Ndelta tautomers are a common feature in the active site of extracellular microbial ribonucleases." *FEBS Lett* **534** (1-3): 197-201.

Plantinga, M. J., Korennykh, A. V., Piccirilli, J. A. and Correll, C. C. (2008). "Electrostatic interactions guide the active site face of a structure-specific ribonuclease to its RNA substrate." *Biochemistry* **47** (34): 8912-8918.

Plantinga, M. J., Korennykh, A. V., Piccirilli, J. A. and Correll, C. C. (2011). "The ribotoxin restrictocin recognizes its RNA substrate by selective engagement of active site residues." *Biochemistry* **50** (14): 3004-3013.

Reiter, Y. and Pastan, I. (1998). "Recombinant Fv immunotoxins and Fv fragments as novel agents for cancer therapy and diagnosis." *Trends Biotechnol* **16** (12): 513-520.

Remacha, M., Jimenez-Diaz, A., Bermejo, B., Rodriguez-Gabriel, M. A., Guarinos, E. and Ballesta, J. P. (1995). "Ribosomal acidic phosphoproteins P1 and P2 are not required for cell viability but regulate the pattern of protein expression in *Saccharomyces cerevisiae*." *Mol Cell Biol* **15** (9): 4754-4762.

Roga, V., Hedeman, L. P. and Olson, B. H. (1971). "Evaluation of mitogillin (NSC-69529) in the treatment of naturally occurring canine neoplasms." *Cancer Chemother Rep* **55** (2): 101-113.

Rossi-Zalaf, L. S. and Alves, S. B. (2006). "Susceptibility of Brevipalpus phoenicis to entomopathogenic fungi." *Exp Appl Acarol* **40** (1): 37-47.

Ruggero, D. and Shimamura, A. (2014). "Marrow failure: a window into ribosome biology." *Blood* **124** (18): 2784-2792.

Santos, C. and Ballesta, J. P. (1994). "Ribosomal protein P0, contrary to phosphoproteins P1 and P2, is required for ribosome activity and *Saccharomyces cerevisiae* viability." *J Biol Chem* **269** (22): 15689-15696.

Shaw, K. E., Davidson, G., Clark, S. J., Ball, B. V., Pell, J. K., Chandler, D. and Sunderland, K. D. (2002). "Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*." *Biological Control* **24** (3): 266-276.

Shi, X., Khade, P. K., Sanbonmatsu, K. Y. and Joseph, S. (2012). "Functional Role of the Sarcin-Ricin Loop of the 23S rRNA in the Elongation Cycle of Protein Synthesis." *J Mol Biol*.

Shim, H. J., Choi, J. Y., Wang, Y., Tao, X. Y., Liu, Q., Roh, J. Y., Kim, J. S., Kim, W. J., Woo, S. D., Jin, B. R. and Je, Y. H. (2013). "NeuroBactrus, a novel, highly effective, and environmentally friendly recombinant baculovirus insecticide." *Appl Environ Microbiol* **79** (1): 141-149.

Sreerama Kumar, P. and Singh, L. (2008). "Enabling mycelial application of *Hirsutella thompsonii* for managing the coconut mite." *Exp Appl Acarol* **46** (1-4): 169-182.

St Leger, R. J. and Wang, C. (2010). "Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests." *Appl Microbiol Biotechnol* **85** (4): 901-907.

Tome-Amat, J., Herrero-Galan, E., Onaderra, M., Martinez-Del-Pozo, A., Gavilanes, J. G. and Lacadena, J. (2015a). "Preparation of an engineered safer immunotoxin against colon carcinoma based on the ribotoxin hirsutellin A." *FEBS J*.

Tome-Amat, J., Ruiz-de-la-Herran, J., Martinez-del-Pozo, A., Gavilanes, J. G. and Lacadena, J. (2015b). "alpha-sarcin and RNase T1 based immunoconjugates: the role of intracellular trafficking in cytotoxic efficiency." *FEBS J* **282** (4): 673-684.

Tome-Amat, J., Olombrada, M., Ruiz-de-la-Herran, J., Perez-Gomez, E., Andradas, C., Sanchez, C., Martinez, L., Martinez-del-Pozo, A., Gavilanes, J. and Lacadena, J. (2015c). "Efficient in vivo antitumor effect of an immunotoxin based on ribotoxin alpha-sarcin in nude mice bearing human colorectal cancer xenografts." *SpringerPlus* **4** (1): 168.

Tomé-Amat, J., Menéndez-Méndez, A., García-Ortega, L., Batt, C. A., Oñaderra, M., Martínez-del-Pozo, A., Gavilanes, J. G. and Lacadena, J. (2012). "Production and characterization of scFvA33T1, an immunoRNase targeting colon cancer cells." *FEBS J* **279** (17): 3022-3032.

Tumer, N. E. and Li, X. P. (2012). "Interaction of ricin and Shiga toxins with ribosomes." *Curr Top Microbiol Immunol* **357**: 1-18.

Turnay, J., Olmo, N., Jimenez, A., Lizarbe, M. A. and Gavilanes, J. G. (1993). "Kinetic Study of the Cytotoxic Effect of alpha-Sarcin, a Ribosome Inactivating Protein from *Aspergillus-Giganteus,* on Tumour Cell Lines - Protein Biosynthesis Inhibition and Cell Binding." *Molecular and Cellular Biochemistry* **122**: 39-47.

Varga, J. and Samson, R. A. (2008). "Ribotoxin genes in isolates of *Aspergillus* section *Clavati*." *Antonie Van Leeuwenhoek* **94** (3): 481-485.

Vey, A., Quiot, J. M., Mazet, I. and McCoy, C. W. (1993). "Toxicity and pathology of crude broth filtarte produced by *Hirsutella thompsonii* var. *thompsonii* in shake culture." *Journal of Invertebrate Pathology* **61**: 131-137.

Viegas, A., Herrero-Galán, E., Oñaderra, M., Macedo, A. L. and Bruix, M. (2009). "Solution structure of hirsutellin A. New insights into the active site and interacting interfaces of ribotoxins." *FEBS J* **276** (8): 2381-2390.

Voorhees, R. M., Schmeing, T. M., Kelley, A. C. and Ramakrishnan, V. (2010). "The mechanism for activation of GTP hydrolysis on the ribosome." *Science* **330** (6005): 835-838.

Warner, J. R. (2015). "Twenty years of ribosome assembly and ribosomopathies." *RNA* **21** (4): 758-759.

Whetstone, P. A. and Hammock, B. D. (2007). "Delivery methods for peptide and protein toxins in insect control." *Toxicon* **49** (4): 576-596.

Woolford, J. L., Jr. and Baserga, S. J. (2013). "Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*." *Genetics* **195** (3): 643-681.

Yang, R. and Kenealy, W. R. (1992). "Regulation of restrictocin production in *Aspergillus restrictus*." *J Gen Microbiol* **138** (7): 1421-1427.

Yang, X., Gerczei, T., Glover, L. T. and Correll, C. C. (2001). "Crystal structures of restrictocin-inhibitor complexes with implications for RNA recognition and base flipping." *Nat Struct Biol* **8** (11): 968-973.

Yang, X. J. and Moffat, K. (1996). "Insights into specificity of cleavage and mechanism of cell entry from the crystal structure of the highly specific *Aspergillus* ribotoxin, restrictocin." *Structure* **4**: 837-852.

ANEXO

Otros artículos que son consecuencia directa del trabajo realizado en esta Tesis Doctoral:

Elías Herrero-Galán, Lucía García-Ortega, **Miriam Olombrada**, Javier Lacadena, Álvaro Martínez del Pozo, José G. Gavilanes y Mercedes Oñaderra (2013) "Hirsutellin A: A paradigmatic example of the insecticidal function of fungal ribotoxins" *Insects* **4**, 339-356.

Jaime Tomé-Amat, **Miriam Olombrada**, Javier Ruiz de la Herrán, Eduardo Pérez Gómez, Clara Andradas, Leopoldo Martínez, Álvaro Martínez del Pozo, José G. Gavilanes y Javier Lacadena (2015) "Efficient *in vivo* antitumor effect of an immunotoxic based on ribotoxin α-sarcin in nude mice bearing human colorrectal cancer xenografts" *SpringerPlus*, **4**:168.

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Review

Hirsutellin A: A Paradigmatic Example of the Insecticidal Function of Fungal Ribotoxins

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Abstract: The fungal pathogen *Hirsutella thompsonii* produces an insecticidal protein named hirsutellin A (HtA), which has been described to be toxic to several species of mites, insect larvae, and cells. On the other hand, on the basis of an extensive biochemical and structural characterization, HtA has been considered to be a member of the ribotoxins family. Ribotoxins are fungal extracellular ribonucleases, which inactivate ribosomes by specifically cleaving a single phosphodiester bond located at the large rRNA. Although ribotoxins were brought to light in the 1960s as antitumor agents, their biological function has remained elusive. Thus, the consideration of hirsutellin A, an insecticidal protein, as a singular ribotoxin recalled the idea of the biological activity of these toxins as insecticidal agents. Further studies have demonstrated that the most representative member of the ribotoxin family, α-sarcin, also shows strong toxic action against insect cells. The determination of high resolution structures, the characterization of a large number of mutants, and the toxicity assays against different cell lines have been the tools used for the study of the mechanism of action of ribotoxins at the molecular level. The aim of this review is to serve as a compilation of the facts that allow identification of HtA as a paradigmatic example of the insecticidal function of fungal ribotoxins.

Keywords: insecticidal proteins; ribotoxins; α-sarcin; hirsutellin; ribonucleases; cytotoxic proteins

1. Introduction

Toxins are molecules affecting vital physiological systems. Many toxins have been explored and developed into drugs for the treatment of many different diseases and others are still under study with the same purpose. But one question to answer is why do organisms need to produce toxins?

All living beings develop several strategies to survive, including a variety of mechanisms for defense, preying, and feeding. Fungi have traditionally been a major source of toxins for study and biotechnological application. They are a rich source of nitrogen and phosphorous and suffer constant attacks by fungivorous animals such as mites, insects, and arthropods [1–3]. Thus, fungi secrete a wide variety of toxins with quite different purposes [4]. Fungal ribotoxins were discovered in the 1960s during a screening program searching for antibiotics and antitumor agents. A protein inhibitory to sarcoma 180 and carcinoma 755 induced in mice was found in the culture filtrates of the mold *Aspergillus giganteus*, isolated from a sample of farm soil. The protein responsible for these effects was named α-sarcin after its anti-sarcoma activity [5]. A few years later it was demonstrated that their mechanism of action was based on their ability to inhibit protein biosynthesis [6,7]. This fact prompted the molecular characterization of ribotoxins as a widespread group of highly specific ribonucleases produced by filamentous fungi, which induce cell death by ribosome inactivation. They are extracellular proteins, which behave as potent inhibitors of protein biosynthesis in almost any organism [8–10]. They cleave one single and unique phosphodiester bond located within the universally conserved sarcin-ricin loop (SRL) of the large rRNA [6,11,12], leading to cell death by apoptosis [13].

Nowadays, four of these ribotoxins have been thoroughly studied and characterized: α-sarcin, the first one to be discovered [5,9]; restrictocin, with similar activities to those of α-sarcin [9,10]; AspF1, a major allergen in *Aspergillus* related diseases [14,15]; and more recently, hirsutellin A, the smallest ribotoxin known. Although ribotoxins were initially discovered as antitumor molecules, further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses [16] and caused the abandonment of their study as toxic molecules.

The biological function of ribotoxins has been a matter of speculation since their discovery. Apart from the generalized idea of being involved in defense or predation, the assignment of an insecticidal function for fungal ribotoxins has been pointed out [17,18]. In this sense, it has been suggested that they could protect the ribotoxin-producing fungi by deterring insect feeding on their phialides [17]. Accordingly, it was proposed that ribotoxin production would be related to fungal protection against arthropods, mainly mites and insects. For example, beetles of the *Carpophilus freemani* species that are able to feed on *Aspergillus restrictus,* the restrictocin-producing organism, could not feed upon this fungus during conidia maturation. On the other hand, beetles were not able to feed on *Aspergillus nidulans* genetically modified to produce the ribotoxin by transforming the fungus with the cDNA of restrictocin placed under the control of the glucoamylase promoter [18]. This protection was not observed in the wild-type fungus in which the genome does not contain any ribotoxin gene [17]. Accordingly, it was also observed that ribotoxins were accumulated on the conidia surface upon maturation of *A. restrictus* [19,20].

In relation to ribotoxin biosynthesis, little is known about the mechanisms by which the producing fungi might protect themselves from their own toxicity. Lamy and Davies [21] suggested that prorestrictocin might be inactive until the protein is processed during secretion, but Yang and Kenealy [22]

showed that neither the leader sequence nor the putative prosequence inhibited the action of the cytotoxin under *in vitro* or *in vivo* conditions. These authors suggest that restrictocin could be sequestered in membrane systems and transported to certain locations or secreted outside. Immunofluorescence studies on the localization of restrictocin in *A. restrictus* support this idea. An alternative hypothesis to the active secretion is that an inactivating protein could bind restrictocin until its liberation, but no evidence exists to support such a system in *A. restrictus*.

The fungal genus *Hirsutella* has over 50 species that are able to colonize a wide variety of insects. Several studies have reported that crude filtrates of a particular species of this genus denominated *Hirsutella thompsonii* were toxic to various groups of arthropods as moth, fly and mosquito larvae, aphids, and mites [23,24], causing their death. This fungus is a specific fungal pathogen of *Acarina* inhabiting citrus and other plants in most subtropical and tropical regions [25] and had been previously developed as a microbial insecticide for use against the mite *Phyllocoptruta oleivora* [26]. The insecticidal action of this invertebrate fungal pathogen has been extensively documented [27,28]

The insecticidal protein HtA was originally detected in 1995 while looking for the effective toxic agents produced by *Hirsutella thompsonii* [28,29]. It has been reported that HtA preparations are highly toxic *in vivo* to the adult citrus rust mite, *Phyllocoptruta oleivora*, the natural host to this parasitic fungus [26]. Moreover, HtA is lethal to *Galleria mellonella* larvae [29] and produces cytopathic effects on certain insect cell lines such as *Spodoptera frugiperda* cells [27]. HtA also inhibits the protein synthesis of the Brome mosaic virus in both rabbit reticulocyte and wheat germ *in vitro* translation system [27].

Recently, evidence has been found that HtA is able to reproduce the specific ribonucleolytic action and other abilities of ribotoxins, and so it has been included as a new member of the α-sarcin/restrictocin family. Although the question of whether HtA is just an exception within the ribotoxin family has been proposed, both its insecticidal and its ribotoxin activities have been clearly demonstrated [26,28–31]. The discovery of this smaller HtA ribotoxin has revived the old proposal that insecticidal ability could be the long searched for natural function of the fungal ribotoxins family. The studies performed comparing the activities of HtA and α-sarcin against insect larvae and cells [31] have shown that HtA could be the demonstration that invertebrate pathogenic activity is the biological function of the ribotoxins family. In this regard, the study of HtA represents an important milestone in the knowledge of the structure, function, and diversity of fungal ribonucleases.

2. Ribotoxins Evolution: Role of HtA

Ribotoxins are an intriguing group of proteins regarding their evolution and structure-function relationships [32,33]. They belong to the barnase superfamily, formed by small ribonucleases consisting of only one polypeptide chain [34]. This superfamily also includes non-toxic unspecific RNases of the T1 family. The high degree of sequence and structural similarity between ribotoxins and T1-like RNases has led to the suggestion that both families could have a common ancestor [32,33]. This similarity includes the active site responsible for the phosphodiesterase activity of these enzymes [35]. RNase U2, produced by the fungus *Ustilago sphaerogena*, stands out as the unspecific fungal extracellular RNase most closely related to ribotoxins [36,37]. It displays 34% sequence identity with the α-sarcin family, and it is 10 residues longer than other T1-like RNases (114 *vs.* 101). Ribotoxins also share, with RNases of the T1 family, their main structural core, but they present a

number of characteristics that make them unique within the whole superfamily. Ribotoxins are basic proteins, and they are around 40 residues longer (140–150 amino acids). The main structural differences between ribotoxins and RNases of the T1 family are the length and arrangement of the non-ordered protein loops and the N-terminal β-hairpin, which are positively charged in ribotoxins. Thus, these regions are supposed to be the determinants of the extra activities of ribotoxins, such as their specificity and cytotoxicity [38]. The ability to enter cells and to display specific ribonucleolytic action against a single phosphodiester bond in the whole ribosome distinguishes ribotoxins from non-cytotoxic relatives of the T1 family. That is to say, ribotoxins are extremely specific ribonucleases when compared to the non-toxic counterparts of the T1 family of fungal extracelular ribonucleases.

Ribotoxins are considered to be naturally engineered proteins that evolved from nontoxic ribonucleases [10]. They exhibit a high degree of identity (above 60%), including two disulphide bridges conserved along the whole family (Figure 1) [9,37,39,40]. Interestingly, HtA shares this characteristic, although it is 20 residues shorter than the other ribotoxins and shows only 25% sequence identity with previously known members of the family [37]. These were the reasons why HtA appeared initially after its discovery as a feasible candidate to be an evolutionary intermediate between T1-like RNases and ribotoxins (Figure 2). However, the further characterization of HtA showed that it maintains all the ribotoxin abilities, proving that these can be accommodated into a shorter amino acid sequence [30,41]. Thus, it has been suggested that HtA could actually be a refined ribotoxin that would have evolved further in order to become smaller and more economical.

Figure 1. (**a**) Alignment of the amino acid sequences of α-sarcin, restrictocin, hirsutellin A, RNase U2, RNase T1, and Barnase. Conserved residues (light grey boxes) in at least four sequences are enlightened, as well as the cysteine residues (dark grey boxes). Residues implicated in the active site are highlighted in black and essential catalytic residues are remarked by (*). Elements of secondary structure are displayed by colors: β-hairpin, (dark blue boxes), residues at the helical portion, (red boxes) and residues in loops 1, 2, 3, 4, and 5 (yellow, green, light blue, pink, and orange boxes, respectively). (**b**) Comparison of the disulfide bridges arrangement in the Barnase superfamily.

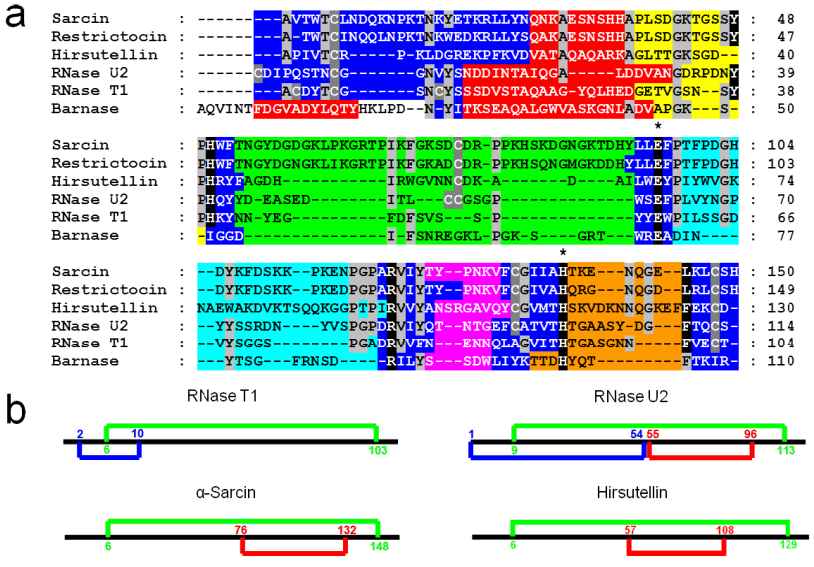
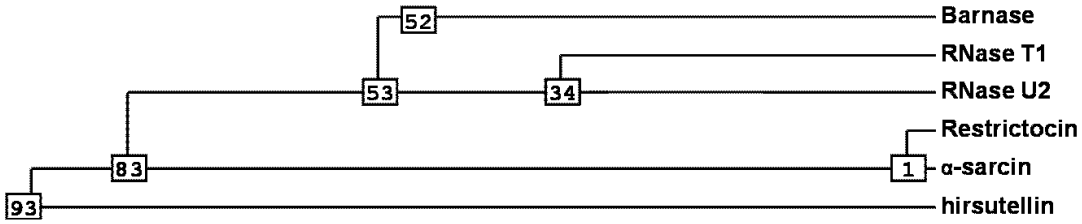


Figure 2. Phylogenetic analysis [42] for the most important members of the Barnase superfamily. Numbers shown in the phylogram are distances corresponding to the amino acid sequence alignment of Figure 1.



3. Structural Features

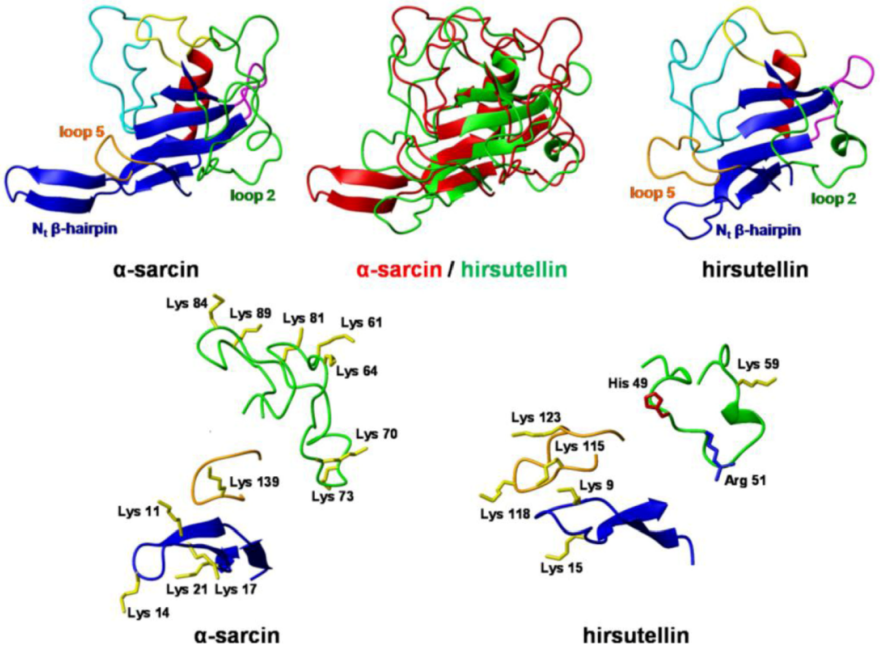
Ribotoxins and non-cytotoxic ribonucleases of the RNase T1 family show high structural homology but different specific activities. Although classic ribotoxins, such as α-sarcin and restrictocin (about 150 amino acids), are larger than non-toxic RNases (about 96–110 amino acids), they share a similar central structure region, including a conserved β-sheet core composed of five strands connected by loops of different lengths. Structural differences between both families are mainly concentrated at these loops and the N-terminal β-hairpin. Consequently, they have been proposed to be the structural determinants responsible for ribotoxin toxic properties [35]. The three-dimensional structures of restrictocin [43] and α-sarcin are known [35,44]. Furthermore, the characterization of a high number of α-sarcin mutants, including bidimensional nuclear magnetic resonance (NMR) studies [45,46], have been the means used to make a very detailed map of its structural and functional properties.

After its discovery as the molecule responsible for the insecticidal activity of the entomopathogenic fungus *Hirsutella thompsonii*, the cDNA of HtA was cloned and sequenced [47]. The corresponding amino acid sequence alignment with other microbial RNases and ribotoxins suggested that the common structural core was also conserved in HtA, the most significant differences being again the length of the loops connecting the α-helical and the β-sheet regions [37]. These loops in HtA were predicted to be longer than the corresponding ones in small microbial RNases, but shorter than those in ribotoxins. In addition, the four cysteine residues involved in two disulfide bridges [35,37] as is the case for α-sarcin, were conserved in HtA (Figure 1b).

From the clone of the cDNA of HtA, plasmid pTac-TacHtA was constructed for the expression of mature HtA in *E. coli* [30]. HtA was purified both from its natural source and also as a recombinant protein. Spectroscopic analysis determined that the fungal protein and the recombinant one were indistinguishable. The exhaustive characterization of both forms of HtA reveals an E coefficient (0.1%, 280 nm, 1 cm) of 2.00 for both proteins [30]. The mid-point of the thermal denaturation transition (T_m) determined by circular dichroism (CD), and differential scanning calorimetry (DSC) was 62 °C. This value was 10 °C higher than that reported for α-sarcin [30] but closer to 61 °C and 59 °C, the T_m values for the ribotoxins AspF1 and restrictocin, respectively [48]. In 2009, the elucidation of the three-dimensional structure of HtA in solution by nuclear magnetic resonance (NMR) [41] confirmed that the overall protein fold of ribotoxins is maintained in this smaller polypeptide chain, but some important differences apart from size-derived variations were observed. The structure consists of one α-helix, one helical turn, and seven β-strands that form a β-sheet and a N-terminal

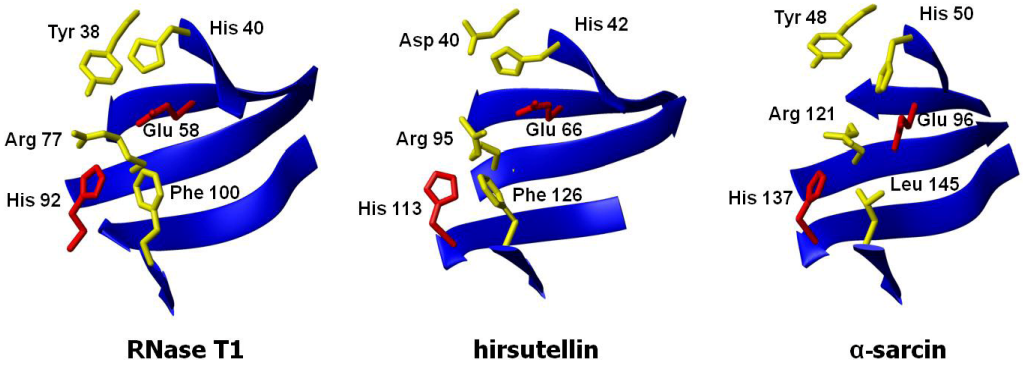
hairpin, with a characteristic α + β fold and a highly positively charged surface. The most relevant structural differences when compared to its larger homolog, α-sarcin, are the shorter lengths of loop 2 and the N-terminal β–hairpin, which is also less positively charged (Figure 3). This truncation and reduced charge of the N-terminal hairpin in HtA may be compensated by the extension and different orientation of its loop 5, which exhibits a higher amount of positively charged residues.

Figure 3. Three-dimensional structures of the ribotoxins α-sarcin and hirsutellin A (HtA) Spatial orientation of the N-terminal β-hairpin and loops 2 and 5 in HtA and α-sarcin. Positively charged residues are depicted. Color code is the same as in Figure 1. The diagrams were generated using MOLMOL [49].



As far as the active site is concerned, that of α-sarcin is well known (Figure 4). Among these residues, His 50, Glu 96, and His 137 form the catalytic triad. The equivalent residues in HtA (His 42, Glu 66, and His 113) have been identified by comparison of its three-dimensional structure with that of other ribotoxins (Figure 4). The essential residues for ribonuclease activity are conserved, but some other features are closer to T1-like RNases (like the presence of a Phe residue instead of α-sarcin's Leu 145) or even completely new in the whole superfamily (an aspartate group at a position equivalent to α-sarcin's Tyr 48, for example) [41]. Several substitution mutants of this region were studied regarding their implication in the functionality of the protein, in order to shed new light on the requirements for ribotoxin activity [50]. Within this idea, a region was found to exhibit significant differences with α-sarcin, related to Trp 71 and Trp 78 in HtA. Studies with single and combined mutants of these two residues revealed that this region seems to be involved in the higher membrane permeabilizing activity of HtA when compared with the other members of the ribotoxins family. The W71/78F mutation in HtA resulted in a loss of cytotoxicity, but maintained the ribonucleolytic specific activity [51]. These residues are not conserved in α-sarcin. It has been postulated that a β-structure region comprising residues 116–139 could be involved in the hydrophobic interaction of α-sarcin with membranes [52].

Figure 4. Three-dimensional structures of the active sites of ribotoxins α-sarcin and hirsutellin A (HtA) and the non-toxic fungal RNase T1. The structures were fitted to the peptide bond atoms of the active site residues of α-sarcin (His 50, Glu 96, and His 137) and RNase T1 (His 40, Glu 58, and His 92) and those at homologous positions in HtA (His 42, Glu 66, and His 113).



4. Functional Properties

It is well known that ribotoxins are cyclizing ribonucleases that exhibit a two-step enzymatic mechanism [9,30,53]. First, they form a 2',3'-cyclic phosphate intermediate via a transphosphorylation reaction and then hydrolyze this intermediate to the corresponding 3'-phosphate [53].

Their toxicity arises from their ability to cross cell membranes and, once inside the cell, impair ribosome function by cleaving a single phosphodiester bond, that between G4325 and A4326 in 28S rRNA (rat ribosome numbering) at the sarcin/ricin loop (SRL) [9]. This cleavage interferes with the function of elongation factors, producing protein biosynthesis inhibition and cell death by apoptosis [13,54]. This highly specific ribonucleolytic activity of ribotoxins has been extensively studied and a wide collection of mutants has been well characterized. Thus, the reaction mechanism, as well as the roles of most of the active site residues, have been elucidated [55–60].

HtA produces the same cleavage as α-sarcin but shows two-fold higher specific activity when assayed against eukaryotic ribosomes. HtA is also able to cleave 35mer synthetic SRL-RNA, liberating 14mer and 21mer oligonucleotides, with identical activity values than those of α-sarcin [30]. Residues forming the catalytic triad of HtA (His 42, Glu 66, and His 137), as well as the outstanding Asp 40 (Figure 4), have been studied in order to shed new light on the requirements for ribotoxin activity. Seven substitution mutants, H42Q, E66Q, and H113Q, as well as double and triple mutants in all possible combinations, were produced and characterized regarding their ribonucleolytic activity and cytotoxicity [50]. Implication of these three residues in the ribotoxin activity of HtA was confirmed, though none of them resulted strictly essential for ribosomal cleavage. An Asp residue (Asp 40 of HtA) is now highlighted as a novelty in this field. It has been clearly established that the catalytic process in ribotoxins is extremely dependent on a precise structural and electrostatic environment of the active site [55,58,59,61]. Studies with mutants D40N and D40N/E66Q demonstrated an important role for Asp 40 in the activity of the protein and revealed a new set of electrostatic interactions quite different from the one described for already known ribotoxins [50], giving to this Asp residue a unique role among the other members of the family.

Although passage through the cell membrane is the first and limiting step for ribotoxin cytotoxicity [62], knowledge about the mechanism of cell entry is scarce. The most relevant data concerning this topic have been obtained for α-sarcin and HtA. This ability to enter cells is also the main functional difference between ribotoxins and their non-cytotoxic relatives microbial RNases from the T1 family. After internalization by endocytosis, ribotoxins reach the cytosol by clathrin-independent transport via acid endosomes and the Golgi [13]. The positively charged surface of ribotoxins seems to be a key factor for this cytotoxicity through interaction with the lipid membrane components since no protein receptor has been found [63–66]. Studies with vesicle-model systems have shown that α-sarcin specifically interacts with acid phospholipid vesicles of phosphatidylserine or phosphatidilglycerol at neutral pH [8,66–68] and promote their aggregation, with the protein acting as a bridge between lipid bilayers [69–71]. This fact would be in agreement with the preference exhibited by ribotoxins for virus-infected or tumor cells, with a higher exposure of acid phospholipids to the extracellular medium due to a loss of symmetry in the plasma membrane [72–76] or a higher content of negatively charged phospholipids, such as phosphatidylserine [77,78]. The involvement in malignant transformation of the enzymes responsible for phosphatidic acid synthesis (diacylglycerol kinases) seems to further support this hypothesis [79–81]. All previous observations are consistent with a membrane interacting mechanism involving an intercalation of the ribotoxins into the lipid matrix, which would promote fusion and permeability changes in the bilayers, processes that would presumably be involved in the ribotoxin passage across the membranes of its target cells [8].

The ability to interact with lipid membranes has been associated with the non-ordered protein loops and the N-terminal β-hairpin of ribotoxins (Figure 3), where the main structural differences between ribotoxins and non-cytotoxic RNases of the T1 family are located. In this regard, deletion of α-sarcin's N-terminal β-hairpin produces an active ribonuclease with altered membrane interaction properties [82,83]. Close to this region, the structure of HtA presents two Trp residues, the above-mentioned Trp 71 and Trp 78, which seem to be taking active part in its higher membrane permeabilizing activity. Studies with single and combined mutants of these two residues provide evidence that cell membrane passage and internalization, as well as substrate specific recognition, require the participation of this region. Additionally, mutant W71/78F has been the first non-cytotoxic but specific ribosome-cleaving ribotoxin obtained to date [51], which has revived the old interest for the potential biomedical application of ribotoxins. One of the classic goals of the study of ribotoxins has been the construction of immunoconjugates with a tumor-specific targeting moiety [84,85], but the non-specific toxicity exhibited by ribotoxins against non-tumoral cells has always been a major concern for researchers in this field. Therefore, HtA W71/78F, which retains the specific ribonucleolytic activity of ribotoxins without their unspecific ability to enter cells, appears as a feasible candidate for the construction of specific and safer immunotoxins, as their *in vivo* cytotoxicity would only be manifested against cells targeted by the conjugated antibody.

5. Insecticidal Activity

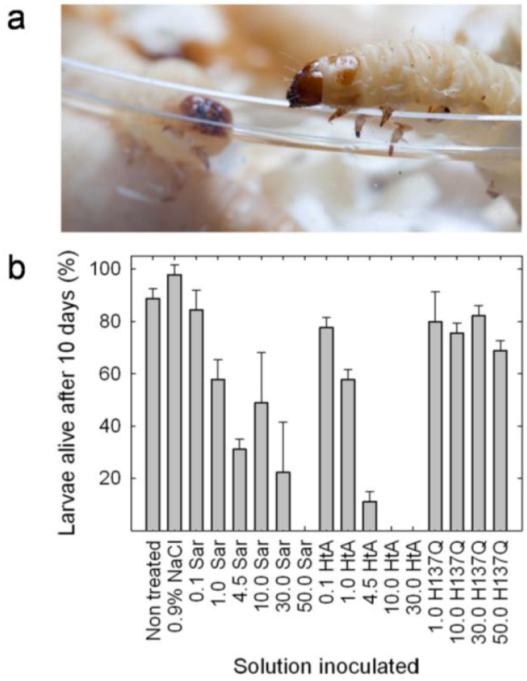
The assignment of an insecticidal function to fungal ribotoxins has been suggested before [17,18]. Several studies have demonstrated that *Hirsutella thompsonii* is able to infect arthropods [23,24]. Under *in vivo* conditions, conidia contact the host, attach to the cuticle, germinate, and penetrate

through it [27]. The potential of *Hirsutella thompsonii* as a biological control agent of the parasitic mite *Varroa destructor*, a honey bee parasite, has also been shown [86,87].

Insect cells have a different plasma membrane composition from mammalian cells, with a higher content of phosphatidylethanolamine and phosphatidylinositol and a significant lower cholesterol/phospholipid ratio [88]. Thus, insect plasma membranes show different permeability. Probably these membranes are thinner and more fluid than those in mammalian cells, being better candidates as ribotoxin targets.

The insecticidal activity of hirsutellin has been well documented [29]. However, no proven data of this activity have been obtained for any of the other members of the ribotoxin family although some studies had suggested that insecticidal activity could be the natural function of ribotoxins [17,18]. Therefore, a study comparing HtA and α-sarcin has been recently performed with the goal of elucidating this idea [31]. The results obtained show that both α-sarcin and hirsutellin A are highly toxic against *G. mellonella* larvae (Figure 5). Injection of α-sarcin or HtA caused larvae death and pupation delay. Virulence was dependent on ribotoxin concentration. Data analysis revealed that there was statistically significant difference between the treatment with α-sarcin and HtA, being HtA more effective in terms of less amount of protein needed to produce the same death levels. Indeed, injection of the catalytically inactive α-sarcin H137Q mutant [56] had an almost negligible effect on survival for identical incubation times and doses, correlating toxicity with the ribonucleolytic activity of these proteins.

Figure 5. (a) *Galleria mellonella* larvae; **(b)** Histogram representing the survival of *G. mellonella* larvae after ten days of being injected with 8 μL of 0.9% NaCl containing different (μM) concentrations of wild-type α-sarcin, a catalytically inactive mutant (H137Q) or wild-type HtA. The initial number of individuals in each group represented was 15. Results correspond to the average of three independent experiments. Bars represent the standard deviation error.



This result suggests that the insecticidal lethal action of ribotoxins is dependent on their highly specific RNase activity. Injection of the toxin produced loss of larvae mobility followed by larvae death, being the differences in larvae survival more evident after 10 days of incubation at 30 ºC [31]. Larvae coloration changed from brownish to dark brown or even black upon death (see complementary video material). These color changes were most probably a consequence of the overactivation of the phenoloxidase cascade, a process that has been proposed as one of the most important defense mechanisms against pathogens in insects [89]. The toxic effect of both ribotoxins was also evident in the observed pupation delay.

Both ribotoxins are also highly toxic against insect cells in culture (Figure 6). Two different insect cell lines have been assayed, *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (High Five). Both α-sarcin and HtA cause a dramatic effect on both inhibition of *in vivo* protein biosynthesis and decreasing cell viability (Table 1). It is remarkable that the IC_{50} values were almost two orders of magnitude smaller than the corresponding values obtained for these ribotoxins against human rhabdomyosarcoma cells [13,30,62], the tumor cell line commonly used as the reference assay for evaluating the antitumoral activity of ribotoxins.

Figure 6. (**a**) Protein biosynthesis inhibition in Sf9 insect cells cultured in the presence of increasing HtA (open circles) or α-sarcin (dark circles) concentrations. Semilogarithmic plots are the average of three different sets of experiments. Bars represent the standard deviation. **(b)** Toxic effect in Sf9 insect cells after the addition of different HtA (grey bars) or α-sarcin concentrations (black bars), expressed in terms of percentage of cell viability after 60 hours of incubation with the protein.

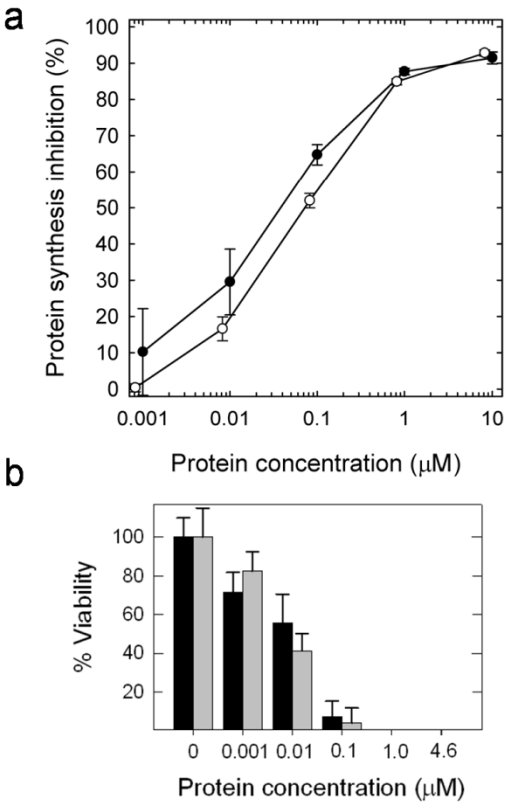


Table 1. IC_{50} values obtained for α-sarcin and HtA when assayed against insect cell lines in culture.

	Viability		Protein biosynthesis inhibition
	Sf9	**High Five**	**Sf9**
α-sarcin	0.010 μM	0.100 μM	0.04 μM
HtA	0.010 μM	0.025 μM	0.07 μM

Moreover, the insect killing activity of both ribotoxins is related to their specific ribonucleolytic action on insect ribosomes. The characteristic activity of ribotoxins is manifested by the production of a 400 nt fragment (α-fragment) upon degradation of the target ribosomes. In fact, the production of α-fragment was observed in treated insect cells. The results revealed the cleavage of 28S rRNA by both ribotoxins with an identical pattern as that described for isolated ribosomes [54]. These data support the idea that the specific ribonucleolytic activity of HtA and α-sarcin is the reason of their toxicity against this insect cell lines. Corroborating that specific ribotoxin action involves the passage of the protein across the membranes of its target cells, both toxins also retained the membrane interaction ability when tested against model vesicles made of insect plasma membrane lipids [31].

Finally, the long studied antitumoral activity of ribotoxins could be a side effect of their insecticidal action and ribotoxins could be considered host-defense proteins and thus included in the new generation of bioinsecticides [90,91].

6. Conclusion

Hirsutellin A (HtA) has been recently demonstrated to be the smallest ribotoxin known to date [30]. It is 20 residues shorter than previously described ribotoxins (130 amino acids compared to 149/150) and exhibits only 25 percent sequence identity to other members of this family. However, it exhibits all the ribotoxin abilities. HtA is able to specifically degrade ribosomes releasing the 400nt α-fragment typically detected after ribotoxin activity. As its larger relatives, it also produces this single cleavage on short synthetic oligonucleotides mimicking the sequence and structure of the SRL [9]. Like these ribotoxins, HtA is highly basic, possesses cytotoxic activity and inhibits protein synthesis [28]. In conclusion, HtA seems to be a more evolved ribotoxin displaying all the functions of ribotoxins in a shorter polypeptide chain [30].

In addition, HtA displays a well-characterized insecticidal biological action [27]. Interestingly, several studies relating ribotoxin expression and conidiophore maturation suggest that ribotoxins may play a defensive role against arthropods like insects or mites [17]. Thus, the characterization of HtA as a ribotoxin may support the theory that they are involved in defense mechanisms for either the producing mold or the plants where they live in symbiosis. We have unequivocally confirmed the insecticidal properties of ribotoxins [31]. First, the interaction of α-sarcin and HtA with insect ribosomes, isolated from *Spodoptera frugiperda* Sf9 cell cultures showed specific ribonucleolytic activity. Second, their insecticidal activity against two different insect cell lines (*Spodoptera frugiperda* Sf9 and *Trichoplusia ni* High five) has been proved and directly related to the catalytic production of the α-fragment. Finally, both ribotoxins exhibit high toxicity when injected at low doses into *G. mellonella* larvae. According to this, it seems reasonable to think that ribotoxins evolved from non-specific RNases driven by the necessity of fungal defense against insects. In this regard, it is very

interesting to consider the fact that insect cell membranes show many of the characteristics of transformed cells and, thus, the insecticidal biological role of ribotoxins could also explain their intriguing antitumoral properties.

In relation to the production of potential insecticidal agents based on ribotoxins, further work must be carried out. Pure restrictocin (1000 p.p.m.) added to the diet of *Carpophilus freemani*, a fungus-feeding beetle, killed 38.5% of larvae in 48 h. Adult *C. freemani* were not affected, but they exhibited an aversion to restrictocin in their diet [17]. In this sense, HtA and α-sarcin could be used as insecticidal agents, but new studies must be established in order to assess a suitable way of toxin administration based on feeding habits. Ribotoxin concentration and digestion control of the proteins ingested should be considered.

Although the unspecific cytotoxic action of ribotoxins could limit their potential use as insecticidal agents, the present accumulation of data about their mechanism of action allow an optimistic view. In this regard, the development of immunotoxins based on these fungal ribonucleases stands out as an alternative in the mid-term future. Nowadays, second generation immunotoxins, based on the fusion of ribtoxins to a single chain containing only the variable domains needed for antigen recognition, have been already obtained [92]. In this sense, a colon cancer-specific immunotoxin, based in α-sarcin, has been recently produced and characterized [84]. These studies also open a new way to solve the potential disadvantages of the application of ribotoxins as insecticides, as conjugation of the toxin to an insect-specific antibody would avoid damage to the beneficiary species. On the other hand, the design of adequate mutants and chimaeric ribotoxins with convenient activities must also be considered.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

References and Notes

1. Berenbaum, M.R.; Eisner, T. Bugs' bugs. *Science* **2008**, *322*, 52–53.
2. Boddy, L.; Jones, T.H. Interactions between basidiomycota and invertebrates. In *Ecology of saprotrophic basidiomycetes*, Proceedings of the British Mycological Society Symposia, Manchester, UK, September 7th 2007; Boddy, L., Frankland, J.C., van West, P., Eds.; Academic Press: Amsterdam, Nederland, 2008; pp. 155–179.
3. Ruess, L.; Lussenhop, J. Trophic interactions of fungi and animals. In *The fungal community—its organization and role in the ecosystem*; Dighton, J., White, F.J., Oudemans, P., Eds.; CRC Press: Boca Raton, FL, USA, 2005; pp. 581–598.

4. Hegedus, N.; Marx, F. Antifungal proteins: More than antimicrobials? *Fungal Biol. Rev.* **2013**, *26*, 132–145.
5. Olson, B.H.; Goerner, G.L. Alpha-sarcin, a new antitumor agent I. Isolation, purification, chemical composition, and the identity of a new amino acid. *Appl. Microbiol.* **1965**, *13*, 314–321.
6. Endo, Y.; Wool, I.G. The site of action of α-sarcin on eukaryotic ribosomes. The sequence at the α-sarcin cleavage site in 28S ribosomal ribonucleic acid. *J. Biol. Chem.* **1982**, *257*, 9054–9060.
7. Schindler, D.G.; Davies, J.E. Specific cleavage of ribosomal RNA caused by α-sarcin. *Nucleic Acids Res.* **1977**, *4*, 1097–1110.
8. Gasset, M.; Mancheño, J.M.; Lacadena, J.; Turnay, J.; Olmo, N.; Lizarbe, M.A.; Martinez-del-Pozo, A.; Oñaderra, M.; Gavilanes, J.G. α-Sarcin, a ribosome-inactivating protein that translocates across the membranes of phospholipid vesicles. *Curr. Topics. Pept. Membr. Res.* **1994**, *1*, 99–104.
9. Lacadena, J.; Alvarez-Garcia, E.; Carreras-Sangra, N.; Herrero-Galan, E.; Alegre-Cebollada, J.; Garcia-Ortega, L.; Oñaderra, M.; Gavilanes, J.G.; Martinez-del-Pozo, A. Fungal ribotoxins: Molecular dissection of a family of natural killers. *FEMS Microbiol. Rev.* **2007**, *31*, 212–237.
10. Martinez-Ruiz, A.; Kao, R.; Davies, J.; Martinez-del-Pozo, A. Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. *Toxicon* **1999**, *37*, 1549–1563.
11. Chan, Y.L.; Endo, Y.; Wool, I.G. The sequence of the nucleotides at the α-sarcin cleavage site in rat 28S ribosomal ribonucleic acid. *J. Biol. Chem.* **1983** *258*, 12768–12770.
12. Endo, Y.; Huber, P.W.; Wool, I.G. The ribonuclease activity of the cytotoxin α-sarcin. The characteristics of the enzymatic activity of alpha-sarcin with ribosomes and ribonucleic acids as substrates. *J. Biol. Chem.* **1983**, *258*, 2662–2667.
13. Olmo, N.; Turnay, J.; Gonzalez de Buitrago, G.; Lopez de Silanes, I.; Gavilanes, J.G.; Lizarbe, M.A. Cytotoxic mechanism of the ribotoxin α-sarcin. Induction of cell death via apoptosis. *Eur. J. Biochem.* **2001**, *268*, 2113–2123.
14. Alvarez-Garcia, E.; Batanero, E.; Garcia-Fernandez, R.; Villalba, M.; Gavilanes, J.G.; Martinez-del-Pozo, A. A deletion variant of the *Aspergillus fumigatus* ribotoxin Aspf1 induces an attenuated airway inflammatory response in a mouse model of sensitization. *J. Investig. Allergol. Clin. Immunol.* **2010**, *20*, 69–75.
15. Arruda, L.K.; Mann, B.J.; Chapman, M.D. Selective expression of a major allergen and cytotoxin, Aspf1, in *Aspergillus fumigatus*. Implications for the immunopathogenesis of *Aspergillus*-related diseases. *J. Immunol.* **1992**, *149*, 3354–3359.
16. Roga, V.; Hedeman, L.P.; Olson, B.H. Evaluation of mitogillin (NSC-69529) in the treatment of naturally occurring canine neoplasms. *Cancer Chemother. Rep.* **1971**, *55*, 101–113.
17. Brandhorst, T.; Dowd, P.F.; Kenealy, W.R. The ribosome-inactivating protein restrictocin deters insect feeding on *Aspergillus restrictus*. *Microbiology* **1996**, *142*, 1551–1556.
18. Brandhorst, T.; Dowd, P.F.; Kenealy, W.R. The effect of fungal ribosome inactivating proteins upon feeding choice in *C. freemani*, and indications of a mutualistic relationship with *A. restrictus*. *Mycopathologia* **2001**, *152*, 155–158.
19. Brandhorst, T.T.; Kenealy, W.R. Production and localization of restrictocin in *Aspergillus restrictus*. *J. Gen. Microbiol.* **1992**, *138*, 1429–1435.

20. Yang, R.; Kenealy, W.R. Regulation of restrictocin production in *Aspergillus restrictus*. *J. Gen. Microbiol.* **1992**, *138*, 1421–1427.
21. Lamy, B.; Davies, J. Isolation and nucleotide sequence of the *Aspergillus restrictus* gene coding for the ribonucleolytic toxin restrictocin and its expression in *Aspergillus nidulans*: The leader sequence protects producing strains from suicide. *Nucleic Acids Res.* **1991**, *19*, 1001–1006.
22. Yang, R.; Kenealy, W.R. Effects of amino-terminal extensions and specific mutations on the activity of restrictocin. *J. Biol. Chem.* **1992**, *267*, 16801–16805.
23. Krasnoff, S.B.; Gupta, S. Identification of the antibiotic phomalactone from the entomopathogenic fungus, *Hirsutella thompsonii* var. *synnematosa*. *J. Chem. Ecol.* **1994**, 293–302.
24. Vey, A.; Quiot, J.M.; Mazet, I.; McCoy, C.W. Toxicity and pathology of crude broth filtarte produced by *Hirsutella thompsonii* var. *thompsonii* in shake culture. *J. Invertebr. Pathol.* **1993**, *61*, 131–137.
25. Samson, R.A.; McCoy, C.W.; O'Donnell, K.L. Taxonomy of the acarine parasite *Hirsutella thompsonii*. *Mycologia* **1980**, *72*, 359–377.
26. Omoto, C.; McCoy, C.W. Toxicity of purified fungal toxin hirsutellin A to the citrus rust mite *Phyllocoptruta oleivora* (ash.). *J. Invertebr. Pathol.* **1998**, *72*, 319–322.
27. Liu, J.C.; Boucias, D.G.; Pendland, J.C.; Liu, W.-Z.; Maruniak, J. The mode of action of hirsutellin A on eukaryotic cells. *J. Invertebr. Pathol.* **1996**, *67*, 224–228.
28. Liu, W.Z.; Boucias, D.G.; McCoy, C.W. Extraction and characterization of the insecticidal toxin hirsutellin A produced by *Hirsutella thompsonii* var. *thompsonii*. *Exp. Mycol.* **1995**, *19*, 254–262.
29. Mazet, I.; Vey, A. Hirsutellin A, a toxic protein produced *in vitro* by *Hirsutella thompsonii*. *Microbiology* **1995**, *141*, 1343–1348.
30. Herrero-Galan, E.; Lacadena, J.; Martinez-del-Pozo, A.; Boucias, D.G.; Olmo, N.; Oñaderra, M.; Gavilanes, J.G. The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. *Proteins* **2008**, *72*, 217–228.
31. Olombrada, M.; Herrero-Galan, E.; Tello, D.; Oñaderra, M.; Gavilanes, J.G.; Martinez-del-Pozo, A.; Garcia-Ortega, L. Fungal extracellular ribotoxins as insecticidal agents. *Insect Biochem. Mol. Biol.* **2013**, *43*, 39–46.
32. Kao, R.; Davies, J. Fungal ribotoxins: A family of naturally engineered targeted toxins? *Biochem. Cell. Biol.* **1995**, *73*, 1151–1159.
33. Lamy, B.; Davies, J.; Schindler, D. The *Aspergillus* ribonucleolytic toxins (ribotoxins). In *Genetically Engineered Toxins*; Frankel, A.E., Ed.; Marcel Dekker Inc.: New York, NY, USA, 1992; pp. 237–258.
34. Aravind, L.; Koonin, E.V. A natural classification of ribonucleases. *Methods Enzymol.* **2001**, *341*, 3–28.
35. Perez-Cañadillas, J.M.; Santoro, J.; Campos-Olivas, R.; Lacadena, J.; Martinez-del-Pozo, A.; Gavilanes, J.G.; Rico, M.; Bruix, M. The highly refined solution structure of the cytotoxic ribonuclease α-sarcin reveals the structural requirements for substrate recognition and ribonucleolytic activity. *J. Mol. Biol.* **2000**, *299*, 1061–1073.
36. Martinez-Ruiz, A.; Garcia-Ortega, L.; Kao, R.; Lacadena, J.; Oñaderra, M.; Mancheño, J.M.; Davies, J.; Martinez-del-Pozo, A.; Gavilanes, J.G. RNase U2 and α-sarcin: A study of relationships. *Methods Enzymol.* **2001**, *341*, 335–351.

37. Martinez-Ruiz, A.; Martinez-del-Pozo, A.; Lacadena, J.; Oñaderra, M.; Gavilanes, J.G. Hirsutellin A displays significant homology to microbial extracellular ribonucleases. *J. Invertebr. Pathol.* **1999**, *74*, 96–97.
38. Martinez-del-Pozo, A.; Gasset, M.; Oñaderra, M.; Gavilanes, J.G. Conformational study of the antitumor protein α-sarcin. *Biochim. Biophys. Acta* **1988**, *953*, 280–288.
39. Lopez-Otin, C.; Barber, D.; Fernandez-Luna, J.L.; Soriano, F.; Mendez, E. The primary structure of the cytotoxin restrictocin. *Eur. J. Biochem.* **1984**, *143*, 621–634.
40. Sacco, G.; Drickamer, K.; Wool, I.G. The primary structure of the cytotoxin α-sarcin. *J. Biol. Chem.* **1983**, *258*, 5811–5818.
41. Viegas, A.; Herrero-Galan, E.; Oñaderra, M.; Macedo, A.L.; Bruix, M. Solution structure of hirsutellin A: new insights into the active site and interacting interfaces of ribotoxins. *FEBS J.* **2009**, *276*, 2381–2390.
42. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680.
43. Yang, X.; Gerczei, T.; Glover, L.; Correll, C.C. Crystal structures of restrictocin-inhibitor complexes with implications for RNA recognition and base flipping. *Nat. Struct. Biol.* **2001**, *8*, 968–973.
44. Campos-Olivas, R.; Bruix, M.; Santoro, J.; Martinez-del-Pozo, A.; Lacadena, J.; Gavilanes, J.G.; Rico, M. H-1 and N-15 nuclear magnetic resonance assignment and secondary structure of the cytotoxic ribonuclease α-sarcin. *Protein Sci.* **1996**, *5*, 969–972.
45. Garcia-Mayoral, M.F.; Garcia-Ortega, L.; Lillo, M.P.; Santoro, J.; Martinez-del-Pozo, A.; Gavilanes, J.G.; Rico, M.; Bruix, M. NMR structure of the noncytotoxic α-sarcin mutant Δ(7-22): The importance of the native conformation of peripheral loops for activity. *Protein Sci.* **2004**, *13*, 1000–1011.
46. Garcia-Mayoral, M.F.; Perez-Cañadillas, J.M.; Santoro, J.; Ibarra-Molero, B.; Sanchez-Ruiz, J.M.; Lacadena, J.; Martinez-del-Pozo, A.; Gavilanes, J.G.; Rico, M.; Bruix, M. Dissecting structural and electrostatic interactions of charged groups in α-sarcin. An NMR study of some mutants involving the catalytic residues. *Biochemistry* **2003**, *42*, 13122–13133.
47. Boucias, D.G.; Farmerie, W.G.; Pendland, J.C. Cloning and sequencing of cDNA of the insecticidal toxin hirsutellin A. *J. Invertebr. Pathol.* **1998,** *72*, 258–261.
48. Garcia-Ortega, L.; Lacadena, J.; Villalba, M.; Rodriguez, R.; Crespo, J.F.; Rodriguez, J.; Pascual, C.; Olmo, N.; Oñaderra, M.; Martinez-del-Pozo, A.; *et al.* Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Aspf1 displaying reduced IgE binding. *FEBS J.* **2005**, *272*, 2536–2544.
49. Koradi, R.; Billeter, M.; Wuthrich, K. MOLMOL: A program for display and analysis of macromolecular structures. *J. Mol. Graph.* **1996**, *14*, 51–55.
50. Herrero-Galan, E.; Garcia-Ortega, L.; Lacadena, J.; Martinez-del-Pozo, A.; Olmo, N.; Gavilanes, J.G.; Oñaderra, M. Implication of an Asp residue in the ribonucleolytic activity of hirsutellin A reveals new electrostatic interactions at the active site of ribotoxins. *Biochimie* **2012**, *94*, 427–433.
51. Herrero-Galan, E.; Garcia-Ortega, L.; Lacadena, J.; Martinez-del-Pozo, A.; Olmo, N.; Gavilanes, J.G.; Oñaderra, M. A non-cytotoxic but ribonucleolytically specific ribotoxin variant: Implication of tryptophan residues in the cytotoxicity of hirsutellin A. *Biol. Chem.* **2012**, *393*, 449–456.

52. Mancheño, J.M.; Gasset, M.; Albar, J.P.; Lacadena, J.; Martínez del Pozo, A.; Oñaderra, M.; Gavilanes, J.G. Membrane interaction of a beta-structure-forming synthetic peptide comprising the 116–139th sequence region of the cytotoxic protein α-sarcin. *Biophys. J.* **1995**, *68*, 2387–2395.
53. Lacadena, J.; Martinez-del-Pozo, A.; Lacadena, V.; Martinez-Ruiz, A.; Mancheño, J.M.; Oñaderra, M.; Gavilanes, J.G. The cytotoxin α-sarcin behaves as a cyclizing ribonuclease. *FEBS Lett.* **1998**, *424*, 46–48.
54. Garcia-Ortega, L.; Alvarez-Garcia, E.; Gavilanes, J.G.; Martinez-del-Pozo, A.; Joseph, S. Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. *Nucleic Acids Res.* **2010**, *38*, 4108–4119.
55. Alvarez-Garcia, E.; Garcia-Ortega, L.; Verdun, Y.; Bruix, M.; Martinez-del-Pozo, A.; Gavilanes, J.G. Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of α-sarcin. *Biol. Chem.* **2006**, *387*, 535–541.
56. Lacadena, J.; Mancheño, J.M.; Martinez-Ruiz, A.; Martinez-del-Pozo, A.; Gasset, M.; Oñaderra, M.; Gavilanes, J.G. Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin. *Biochem. J.* **1995**, *309*, 581–586.
57. Lacadena, J.; Martinez-del-Pozo, A.; Martinez-Ruiz, A.; Perez-Cañadillas, J.M.; Bruix, M.; Mancheño, J.M.; Oñaderra, M.; Gavilanes, J.G. Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin α-sarcin. *Proteins* **1999**, *37*, 474–484.
58. Masip, M.; Garcia-Ortega, L.; Olmo, N.; Garcia-Mayoral, M.F.; Pérez-Cañadillas, J.M.; Bruix, M.; Oñaderra, M.; Martinez-del-Pozo, A.; Gavilanes, J.G. Leucine 145 of the ribotoxin α-sarcin plays a key role for determining the specificity of the ribosome-inactivating activity of the protein. *Protein Sci.* **2003**, *12*, 161–169.
59. Masip, M.; Lacadena, J.; Mancheño, J.M.; Oñaderra, M.; Martinez-Ruiz, A.; Martinez-del-Pozo, A.; Gavilanes, J.G. Arginine 121 is a crucial residue for the specific cytotoxic activity of the ribotoxin α-sarcin. *Eur. J. Biochem.* **2001**, *268*, 6190–6196.
60. Siemer, A.; Masip, M.; Carreras, N.; Garcia-Ortega, L.; Oñaderra, M.; Bruix, M.; Martinez-del-Pozo, A.; Gavilanes, J.G. Conserved asparagine residue 54 of α-sarcin plays a role in protein stability and enzyme activity. *Biol. Chem.* **2004**, *385*, 1165–1170.
61. Pérez-Cañadillas, J.M.; Campos-Olivas, R.; Lacadena, J.; Martinez-del-Pozo, A.; Gavilanes, J.G.; Santoro, J.; Rico, M.; Bruix, M. Characterization of pK(a) values and titration shifts in the cytotoxic ribonuclease α-sarcin by NMR. Relationship between electrostatic interactions, structure, and catalytic function. *Biochemistry* **1998**, *37*, 15865–15876.
62. Turnay, J.; Olmo, N.; Jimenez, A.; Lizarbe, M.A.; Gavilanes, J.G. Kinetic study of the cytotoxic effect of α-sarcin, a ribosome inactivating protein from *Aspergillus giganteus*, on tumour cell lines: Protein biosynthesis inhibition and cell binding. *Mol. Cell. Biochem.* **1993**, *122*, 39–47.
63. Di Donato, A.; Cafaro, V.; Dalessio, G. Ribonuclease A can be transformed into a dimeric ribonuclease with antitumor activity. *J. Biol. Chem.* **1994**, *269*, 17394–17396.
64. Ilinskaya, O.N.; Dreyer, F.; Mitkevich, V.A.; Shaw, K.L.; Pace, C.N.; Makarov, A.A. Changing the net charge from negative to positive makes ribonuclease Sa cytotoxic. *Protein Sci.* **2002**, *11*, 2522–2525.

65. Vatzaki, E.H.; Allen, S.C.; Leonidas, D.D.; Trautwein-Fritz, K.; Stackhouse, J.; Benner, S.A.; Acharya, K.R. Crystal structure of a hybrid between ribonuclease A and bovine seminal ribonuclease—the basic surface, at 2.0 Å resolution. *Eur. J. Biochem.* **1999**, *260*, 176–182.
66. Carreras-Sangra, N.; Alvarez-Garcia, E.; Herrero-Galan, E.; Tome-Amat, J.; Lacadena, J.; Alegre-Cebollada, J.; Oñaderra, M.; Gavilanes, J.G.; Martinez-del-Pozo, A. The therapeutic potential of fungal ribotoxins. *Curr. Pharm. Biotechnol.* **2008**, *9*, 153–160.
67. Gasset, M.; Martinez-del-Pozo, A.; Oñaderra, M.; Gavilanes, J.G. Study of the interaction between the antitumour protein α-sarcin and phospholipid vesicles. *Biochem. J.* **1989**, *258,* 569–575.
68. Gasset, M.; Oñaderra, M.; Martinez-del-Pozo, A.; Schiavo, G.-P.; Laynez, J.; Usobiaga, P.; Gavilanes, J.G. Effect of the antitumour protein α-sarcin on the thermotropic behaviour of acid phospholipid vesicles. *Biochim. Biophys. Acta* **1991**, *1068*, 9–16.
69. Gasset, M.; Oñaderra, M.; Goormaghtigh, E.; Gavilanes, J.G. Acid phospholipid vesicles produce conformational changes on the antitumour protein α-sarcin. *Biochim. Biophys. Acta* **1991**, *1080*, 51–58.
70. Gasset, M.; Oñaderra, M.; Thomas, P.G.; Gavilanes, J.G. Fusion of phospholipid vesicles produced by the anti-tumour protein α-sarcin. *Biochem. J.* **1990**, *265*, 815–822.
71. Mancheño, J.M.; Gasset, M.; Lacadena, J.; Ramon, F.; Martinez-del-Pozo, A.; Oñaderra, M.; Gavilanes, J.G. Kinetic study of the aggregation and lipid mixing produced by α-sarcin on phosphatidylglycerol and phosphatidylserine vesicles: Stopped-flow light scattering and fluorescence energy transfer measurements. *Biophys. J.* **1994** , *67*, 1117–1125.
72. Bergelson, L.D.; Dyatlovitskaya, E.V.; Torkhovskaya, T.I.; Sorokina, I.B.; Gorkova, N.P. Phospholipid composition of membranes in the tumor cell. *Biochim. Biophys. Acta* **1970**, *210*, 287–298.
73. Carrasco, L.; Esteban, M. Modification of membrane permeability in vaccinia virus-infected cells. *Virology* **1982**, *117*, 62–69.
74. Fernandez-Puentes, C.; Carrasco, L. Viral infection permeabilizes mammalian cells to protein toxins. *Cell* **1980**, *20*, 769–775.
75. Orntoft, T.F.; Vestergaard, E.M. Clinical aspects of altered glycosylation of glycoproteins in cancer. *Electrophoresis* **1999**, *20*, 362–371.
76. Ran, S.; Downes, A.; Thorpe, P.E. Increased exposure of anionic phospholipids on the surface of tumor blood vessels. *Cancer Res.* **2002**, *62*, 6132–6140.
77. Connor, J.; Bucana, C.; Fidler, I.J.; Schroit, A.J. Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: Quantitative assessment of outer-leaflet lipid by prothrombinase complex formation. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 3184–3188.
78. Zachowsky, A. Phospholipids in animal eukaryotic membranes: Transverse asymmetry and movement. *Biochem. J.* **1993**, *294*, 1–14.
79. Filigheddu, N.; Cutrupi, S.; Porporato, P.E.; Riboni, F.; Baldanzi, G.; Chianale, F.; Fortina, E.; Piantanida, P.; De Bortoli, M.; Vacca, G.; *et al.* Diacylglycerol kinase is required for HGF-induced invasiveness and anchorage-independent growth of MDA-MB-231 breast cancer cells. *Anticancer Res.* **2007**, *27*, 1489–1492.
80. Griner, E.M.; Kazanietz, M.G. Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer* **2007**, *7*, 281–294.

81. Merida, I.; Avila-Flores, A.; Merino, E. Diacylglycerol kinases: At the hub of cell signalling. *Biochem. J.* **2008**, *409*, 1–18.
82. Garcia-Ortega, L.; Lacadena, J.; Mancheño, J.M.; Oñaderra, M.; Kao, R.; Davies, J.; Olmo, N.; Martinez-del-Pozo, A.; Gavilanes, J.G. Involvement of the N-terminal β-hairpin of the *Aspergillus* ribotoxins on the interaction with membranes and nonspecific ribonuclease activity. *Protein Sci.* **2001**, *10*, 1658–1668.
83. Garcia-Ortega, L.; Masip, M.; Mancheño, J.M.; Oñaderra, M.; Lizarbe, M.A.; Garcia-Mayoral, M.F.; Bruix, M.; Martinez-del-Pozo, A.; Gavilanes, J.G. Deletion of the N-terminal β-hairpin of the ribotoxin α-sarcin produces a nontoxic but active ribonuclease. *J. Biol. Chem.* **2002,** *277*, 18632–18639.
84. Carreras-Sangra, N.; Tome-Amat, J.; Garcia-Ortega, L.; Batt, C.A.; Oñaderra, M.; Martinez-del-Pozo, A.; Gavilanes, J.G.; Lacadena, J. Production and characterization of a colon cancer-specific immunotoxin based on the fungal ribotoxin α-sarcin. *Protein Eng. Des. Sel.* **2012**, *25*, 425–435.
85. Tome-Amat, J.; Menendez-Mendez, A.; Garcia-Ortega, L.; Batt, C.A.; Oñaderra, M.; Martinez-del-Pozo, A.; Gavilanes, J.G.; Lacadena, J. Production and characterization of scFvA33T1, an immunoRNase targeting colon cancer cells. *FEBS J.* **2012**, *279*, 3022–3032.
86. Kanga, L.H.; James, R.R.; Boucias, D.G. *Hirsutella thompsonii* and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor*, a honey bee parasite. *J. Invertebr. Pathol.* **2002**, *81*, 175–184.
87. Peng, C.Y.; Zhou, X.; Kaya, H.K. Virulence and site of infection of the fungus *Hirsutella thompsonii* to the honey bee ectoparasitic mite, *Varroa destructor*. *J. Invertebr. Pathol.* **2002**, *81*, 185–195.
88. Marheineke, K.; Grunewald, S.; Christie, W.; Reilander, H. Lipid composition of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) insect cells used for baculovirus infection. *FEBS Lett.* **1998**, *441*, 49–52.
89. Soderhall, K.; Cerenius, L. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* **1998**, *10*, 23–28.
90. Craik, D.J. Host-defense activities of cyclotides. *Toxins* **2012**, *4*, 139–156.
91. Windley, M.J.; Herzig, V.; Dziemborowicz, S.A.; Hardy, M.C.; King, G.F.; Nicholson, G.M. Spider-venom peptides as bioinsecticides. *Toxins* **2012**, *4*, 191–227.
92. Rathore, D.; Batra, J.K. Cytotoxic activity of ribonucleolytic toxin restrictocin-based chimeric toxins targeted to epidermal growth factor receptor. *FEBS Lett.* **1997**, 407, 275–279.

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Efficient *in vivo* antitumor effect of an immunotoxin based on ribotoxin α-sarcin in *nude* mice bearing human colorectal cancer xenografts

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Abstract

Tagging of RNases, such as the ribotoxin α-sarcin, with the variable domains of antibodies directed to surface antigens that are selectively expressed on tumor cells endows cellular specificity to their cytotoxic action. A recombinant single-chain immunotoxin based on the ribotoxin α-sarcin (IMTXA33αS), produced in the generally regarded as safe (GRAS) yeast *Pichia pastoris*, has been recently described as a promising candidate for the treatment of colorectal cancer cells expressing the glycoprotein A33 (GPA33) antigen, due to its high specific and effective cytotoxic effect on *in vitro* assays against targeted cells. Here we report the *in vivo* antitumor effectiveness of this immunotoxin on *nude* mice bearing GPA33-positive human colon cancer xenografts. Two sets of independent assays were performed, including three experimental groups: control (PBS) and treatment with two different doses of immunotoxin (50 or 100 μg/ injection) (n = 8). Intraperitoneal administration of IMTXA33αS resulted in significant dose-dependent tumor growth inhibition. In addition, the remaining tumors excised from immunotoxin-treated mice showed absence of the GPA33 antigen and a clear inhibition of angiogenesis and proliferative capacity. No signs of immunotoxin-induced pathological changes were observed from specimens tissues. Overall these results show efficient and selective cytotoxic action on tumor xenografts, combined with the lack of severe side effects, suggesting that IMTXA33αS is a potential therapeutic agent against colorectal cancer.

Keywords: Immunotoxin; *in vivo* antitumor effectiveness; Colorectal cancer; GPA33; Ribotoxin α-sarcin

Introduction

Colon cancer is among the most deadly ones with a significant worldwide incidence. Its treatment by immunotherapy is becoming relatively successful with three monoclonal antibodies already approved for clinical use (Eng 2010; Tol and Punt 2010; Sliwkowski and Mellman 2013). However, its late diagnosis and metastatic progression makes the development of more efficient drugs necessary. In this scenario, immunotoxins are highly specific therapeutic agents that hold promise as antitumoral agents. They combine the specificity of an antibody fragment with the potency of a toxin, causing death of the target cells (Pastan et al. 2007; Dougan and Dranoff 2009).

Immunotherapeutic approaches using antibodies have been widely explored against a variety of tumors but an effective treatment of solid tumors still remains as a problem because therapeutic antibodies must diffuse into tumors through a disordered vasculature and against a hydrostatic pressure gradient (Jain 2001; Dienstmann et al. 2012). Immunotoxins design has greatly evolved focused on the targeting domain, mainly towards a better penetration in solid tumors or an increase of immunotoxins stability or functionality (Onda et al. 2011; Gehlsen et al. 2012). Because low–molecular weight antibody fragments have been shown to have better tumor diffusion properties, single-chain variable fragments (scFv) have

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been favored to deliver protein-based toxins to cancer cells (Madhumati and Verma 2012; Sapra and Shor 2013).

GPA33 is an extensively studied membrane antigen (Heath et al. 1997) overexpressed in 95% of primary or metastatic colorectal cancers and absent in most of any other tissue, tumoral or not. Due to its features, GPA33 represents an ideal target for immunotoxins aimed against colon cancer cells (Scott et al. 2005; Ackerman et al. 2008). In fact, the three mAbs approved for colon cancer immunotherapy, bevacizumab and cetuximab or panitumumab, are not completely specific for target colon cancer, as they recognize the vascular endothelial growth factor (VEGF) or the epidermal growth factor receptor (EGFR), respectively (Sliwkowski and Mellman 2013).

Different A33 humanized monoclonal antibody (huA33) based constructions against GPA33 antigen have been described, including several clinical assays with radioimmunoconjugates using the whole antibody molecule (Welt et al. 2003; Scott et al. 2005; Almqvist et al. 2006), recombinant scFv designs for Antibody-Directed-Enzyme-Prodrug-Therapy (ADEPT) (Coelho et al. 2007; Panjideh et al. 2008) or, more recently, preclinical assays with immuno-targeted gold-iron oxide hybrid nanoparticles for therapeutic strategies (Kirui et al. 2013).

Regarding the toxic moiety, human or fungal ribonucleases (RNases) have become a new alternative for their use in this domain as opposed to the commonly used ricin and *Diphtheria* or *Pseudomonas* toxins (Ardelt 2013), which in many cases show immunogenic reactivity or undesirable side effects (Frankel et al. 2000; Schindler et al. 2001; Onda et al. 2011). In this sense, ribotoxins are cytotoxic fungal extracellular RNases with α-sarcin as its most outstanding member (Lacadena et al. 2007; Olombrada et al. 2014a). They behave as potent inhibitors of protein biosynthesis due to its highly specific ribonucleolytic activity, which cleaves a single phosphodiester bond of the larger molecule of rRNA located at a universally conserved site, known as the sarcin/ricin loop (SRL), leading to cell death by apoptosis (Schindler and Davies 1977; Lacadena et al. 1999; Olmo et al. 2001; García-Ortega et al. 2010; Olombrada et al. 2014b)

Ribotoxins have several advantages for their use as immunotoxins toxic moiety. Namely, their small size, high thermostability, poor immunogenicity, resistance to proteases and highly efficient ability to inactivate ribosomes (Rathore and Batra 1996; Goyal and Batra 2000; Lacadena et al. 2007; Carreras-Sangrà et al. 2008). Thus, different ribotoxins have been used before as components of immunotoxins, and assayed *in vitro* (Orlandi et al. 1988; Wawrzynczak et al. 1991; Better et al. 1992; Rathore et al. 1997; Goyal and Batra 2000).

Recently, we have reported the first ribotoxin-based-scFv recombinant immunotoxin directed against human colorectal cancer cells by the fusion of humanized scFvA33 and α-sarcin (IMTXA33αS) (Carreras-Sangrà et al. 2012). Incubation with nanomolar concentrations resulted in a very high specific and effective cytotoxicity against targeted cells in *in vitro* assays.

The results herein presented, in terms of its *in vivo* specific antitumor effect using *nude* mice harbouring colon cancer xenografts, reveal a great antitumor effectiveness with a strong inhibition of tumor growth, angiogenesis and proliferative properties.

Results

IMTXA33αS inhibits protein synthesis in vitro

IMTXA33αS production and structural and *in vitro* functional characterization have been previously reported (Carreras-Sangrà et al. 2012). IMTXA33αS shows a highly specific and effective cytotoxicity against colon cancer target cells (SW1222 or LIM1215) overexpressing the GPA33 antigen, with IC_{50} (protein concentration inhibiting 50% of protein synthesis) values of 30 and 70 nM, respectively. Unlike, free wild type α-sarcin exhibits no difference between negative and positive antigen cells, with IC_{50} values of ≥1 μM (Carreras-Sangrà et al. 2012). Moreover, when we analyzed the cytotoxicity of the scFvA33 alone against GPA33-positive SW1222 cells cultures IC_{50} value was too high to be measurable (Table 1). Thus, as expected, the scFvA33 target was not able to induce death of the targeted cells, unless fused to α-sarcin as part of the immunotoxin.

IMTXA33αS reduces tumor growth in vivo

To study the *in vivo* effect of the immunotoxin, the development of solid tumors was induced in *nude* mice by inoculation of SW1222 cells, as GPA33-positive cell model, in the rear right flank. SW1222-xenografts started to appear approximately seven days after cell injection with a significant and rapidly heterogeneous growth as previously described (Barendswaard et al. 2001). Mice with palpable tumors of 50–100 mm^3 of volume were challenged with immunotoxin treatment as described in Materials and Methods. Wild type α-sarcin and scFvA33 were also included as controls in the *in vivo* assay (Figure 1A). As expected, scFvA33 administration did not affect

Table 1 *In vitro* cytotoxicity of IMTXA33αS, wild type α-sarcin and scFvA33

	GPA33 (+)		GPA33 (−)	
	SW1222	LIM1215	HT29	A431
IMTXA33αS	0.03*	0.07	> > 1.00*	> > 1.00*
α-Sarcin	0.8*	> 1.00	> > 1.00*	1.00*
scFvA33	NM	NM	NM	NM

*, (Carreras-Sangrà et al. 2012); NM, too high to be measurable.
IC_{50} (protein concentration inhibiting 50% of protein biosynthesis) (μM) obtained for the cell lines assayed, after 72 h of incubation with the different proteins.

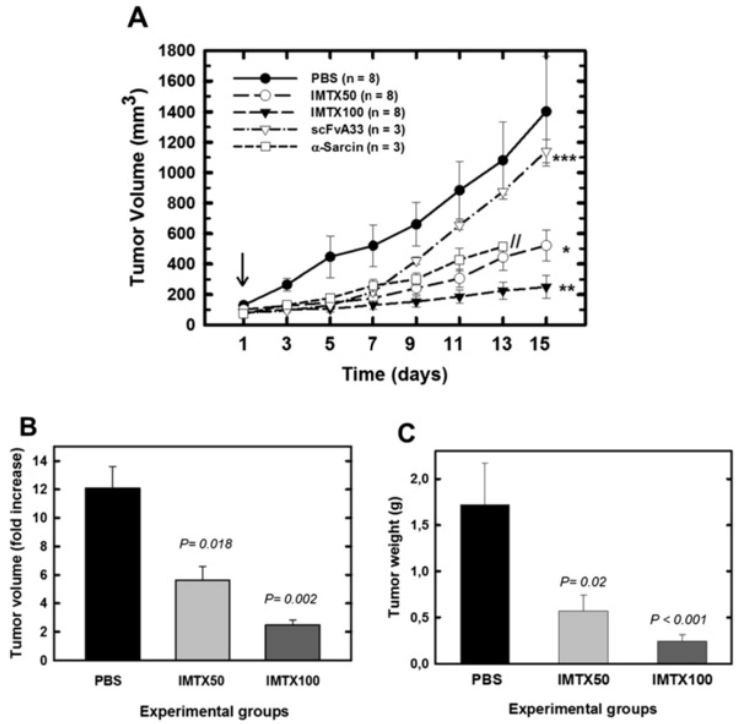

Figure 1 Immunotoxin inhibits colorectal tumor growth *in vivo*. A) Time course of the tumor volume progression of SW1222-derived xenografts non-treated (PBS) or treated with 50 or 100 μg IMTXA33αS (IMTX50 or IMTX100, respectively), 40 μg wild type α-sarcin or 61 μg scFvA33, per injection. α-Sarcin and scFvA33 doses were equivalent to the highest dose of immunotoxin used (IMTX100), 2.2 nanomoles in each case. The arrow indicates the beginning of treatment Doses were given every 48 hours. *, $p < 0.05$; **, $p < 0.01$; ***, $p > 0.05$ *vs* vehicle treated tumors. //, indicates suspension of treatment. **B)** Growth and **C)** weight measurements of excised tumors of non-treated (PBS) or after *in vivo* treatment with IMTXA33αS.

tumor growth except from a slight delay, which was not significant from a statistical point of view. By contrast, treatment with wild type α-sarcin produced a significant inhibition of tumor growth, but treatment had to be suspended because of toxicity in mice, which showed skin disorders, weight loss and decreased mobility.

We then analyzed the effect of two doses of immunotoxin (IMTX50 and IMTX100 experimental groups) on tumor progression compared with the vehicle group (PBS) (Figure 1A). Treatment with the immunotoxin strongly slowed down tumor growth in a dose-dependent manner. At the end of the treatment, tumor volume in animals challenged with IMTX50 and IMTX100 was reduced up to 3 to 6 times, respectively, as compared to vehicle-treated animals (Figure 1B). Similar results were obtained when tumor weight was considered (Figure 1C). Tumors were analyzed by H&E staining showing the presence of healthy tumor cells and well-vascularized tissues with no apparent cell damage in non-treated mice, as opposed to the cell damage and necrotic features observed in the immunotoxin-treated tumor cells (data not shown).

IMTXA33αS reduces GPA33 antigen expression, angiogenesis and cancer cell proliferation in vivo

The analysis of the tumors revealed that the percentage of GPA33-positive cells in the tumors showed a large decrease after treatment with the immunotoxin, due to death of target cells (Figure 2), with less than 10% of positive area for the highest dose. Thus, most of the remaining cells from these tumors lost the GPA33 antigen and, presumably, the tumor aggressiveness.

Interestingly, when the animals were sacrificed and the remaining tumors were excised significant differences in their morphologic features were evident. Tumors from the non-treated group were solid and well vascularized. On the other hand some of the IMTX100-treated mice tumors were more whitish, squishy and cyst-like (Figure 3A). This colour differences were suggestive of effects on the angiogenic process. In fact, tumor vascularization was impaired by the immunotoxin, as the number of blood vessels was smaller, as determined by CD31 staining (Figure 3B), confirming what was observed in the macroscopic analysis.

The proliferative potential of cancer cells present in the tumors was also analyzed. A significant reduction of

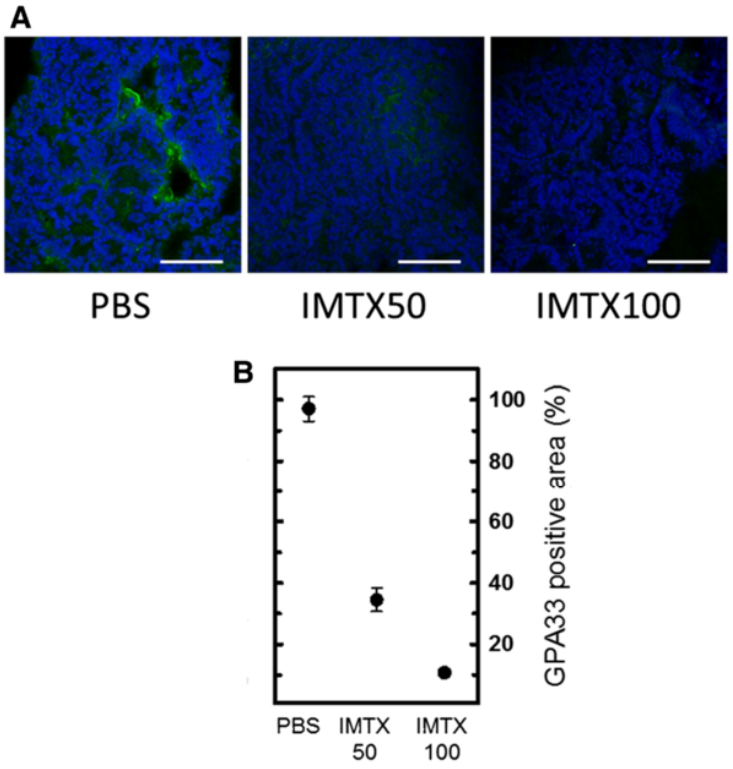

Figure 2 IMTXA33αS-treated tumors show lack of GPA33 antigen *in vivo*. A) GPA33-positive cells (green). Scale bars: 50 μm. **B)** Quantification of GPA33-positive cells. Statistical analysis of IMTX50 or IMTX100-treated tumors *vs* vehicle-treated tumors rendered $p < 0.001$.

this potential was again observed in immunotoxin-treated tumors, as indicated by the dose dependent decrease in the number of Ki67-positive cells (Figure 4A and C). Moreover, immunotoxin administration increased the number of active caspase 3-positive cells, indicating tumor cells death by apoptosis (Figure 4B and C).

IMTXA33αS does not produce adverse side effects

Growth of the mice was not affected by treatment with IMTXA33αS in any of the doses assayed, considering their weight (Figure 5A) and external appearance. In this sense, regardless of the group to which they belonged, weight was increased in about 20% at the end of the treatment.

None of the mice showed histomorphologic changes in a macroscopic analysis resulting from immunotoxin treatment. Moreover no significant changes were observed in the histological analysis by H&E staining in any of the organs analyzed, regardless of the experimental group they belonged (Figure 5B).

Discussion

ScFv-immunotoxins have been lately used for experimental therapeutic approaches due to its more favourable penetration and diffusion features in solid tumor tissues. Within this idea, we have recently reported the high specificity and effectiveness on *in vitro* assays of two recombinant scFv-immunoconjugates, based on the fungal RNases α-sarcin and RNase T1, against colon cancer targeted cells (Carreras-Sangrà et al. 2012; Tomé-Amat et al. 2012).

In this work we move one step further and demonstrate the potential of IMTXA33αS for *in vivo* applications, using SW1222-induced xenografts, as a model of GPA33-positive colon cancer. The results have shown a clear inhibition effect on tumor growth accompanied by a significant decrease in the proliferative and angiogenic capacities of the treated tumors, as determined by Ki67 and CD31 labeling. The inhibition of protein biosynthesis in immunotoxin-treated tumors, due to α-sarcin specific RNase activity, led to apoptosis causing cell death, as determined by active-caspase 3 labeling. This mechanism is a well-known feature of the ribotoxic action of this toxin (Olmo et al. 2001; Lacadena et al. 2007).

Furthermore, treatment with the highest dose of immunotoxin resulted in a residual tumor mass, lacking the GPA33 antigen and with a cyst-like appearance. GPA33 belongs to the immunoglobulin superfamily with homology to tight junction-associated proteins. It's closest homologous include the junction adhesion molecules (JAM) or the CEA-related cell adhesion

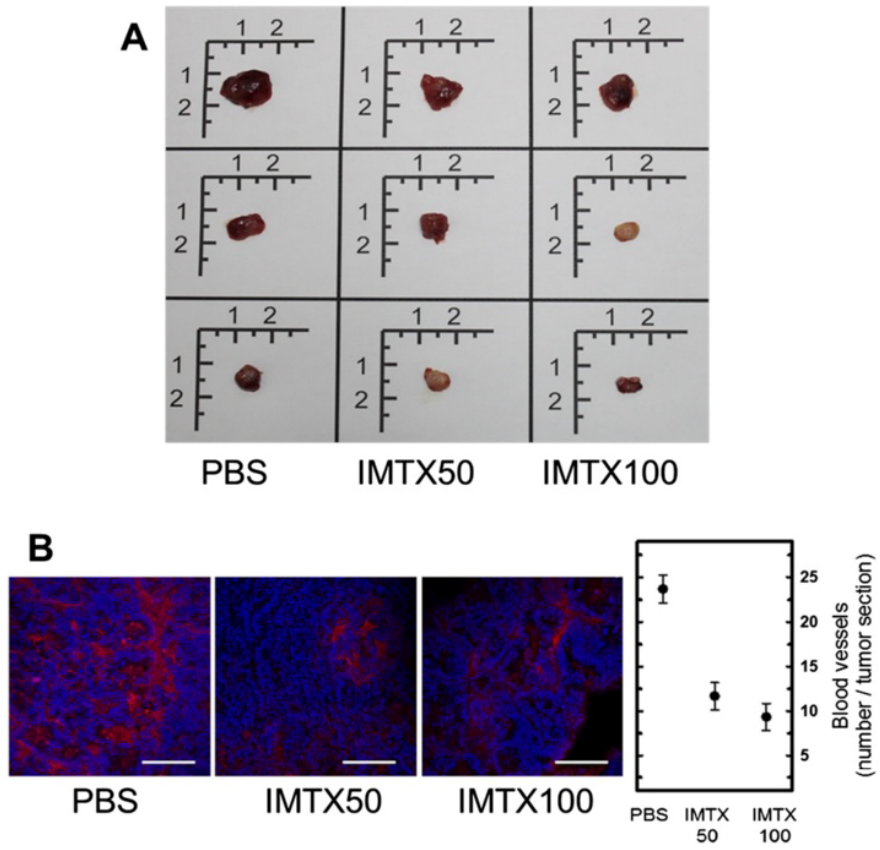

Figure 3 IMTXA33αS impaired angiogenesis on *in vivo* treated tumors. A) Representative images of the excised remaining tumors of non-treated (PBS) or treated (IMTX50, IMTX100) experimental groups. Scale is in centimeters. Each column shows three examples from each group. **B)** CD31-positive cells (red). Scale bars: 60 μm. Quantification of the number of blood vessels is shown in the corresponding graph. Statistical analysis of IMTX50 or IMTX100-treated tumors *vs* vehicle-treated tumors rendered p <0001.

molecules, among others (Ackerman et al. 2008), which are involved in tumor proliferation (Goetsch et al. 2013) or tumor angiogenesis (Kuespert et al. 2006), respectively. Thus, the absence of the GPA33 antigen after treatment with IMTXA33αS would be consistent with the observed anti-angiogenic effect and the decrease in the proliferative potential of the residual tumor mass. These results would suggest a correlation between GPA33-expression level and aggressiveness or the tumor, as previously described for JAM in relation to poor prognosis of breast cancer (McSherry et al. 2009).

Moreover, along with the high antitumor effectiveness of IMTXA33αS, adverse side effects were not observed at the histological level or in the development of the treated mice. Thus the scFvA33 target domain directs α-sarcin to the target cells avoiding the nonspecific toxicity shown in the *in vivo* assay with free wild type α-sarcin (Figure 1). Similar effects have been previously described for free wild type α-sarcin and other ribotoxins, like mitogillin, in clinical trials (Goldin et al. 1966; Roga et al. 1971). For example, neurologic symptoms, gastrointestinal toxicity or frequent skin rash have been previously described for free α-sarcin *in vivo* assays with mice (Goldin et al. 1966). Mitogillin has also been evaluated by repeated-dose toxicity tests performed in mice, dogs and monkeys (Roga et al. 1971). In the particular case of mice that received 0.2 mg/kg/day, only two of ten mice survived the test period, and all of them showed weight loss and organs injury (Roga et al. 1971). Similar effects were described in the other trials (Roga et al. 1971). This explains why in the 1970s these proteins were abandoned as promising antitumoral agents. However, none of these effects have appeared in the immunotoxin-treated mice, despite having used fivefold higher doses of immunotoxin (in nmoles) than those described for the ribotoxin. A result which again favours the interpretation, that the immunotoxin action is highly specific *in vivo*. Thus, IMTXA33αS shows both high efficacy and specificity against colon cancer tumors on *in vivo* assays with undetectable unwanted side-effects. Further steps

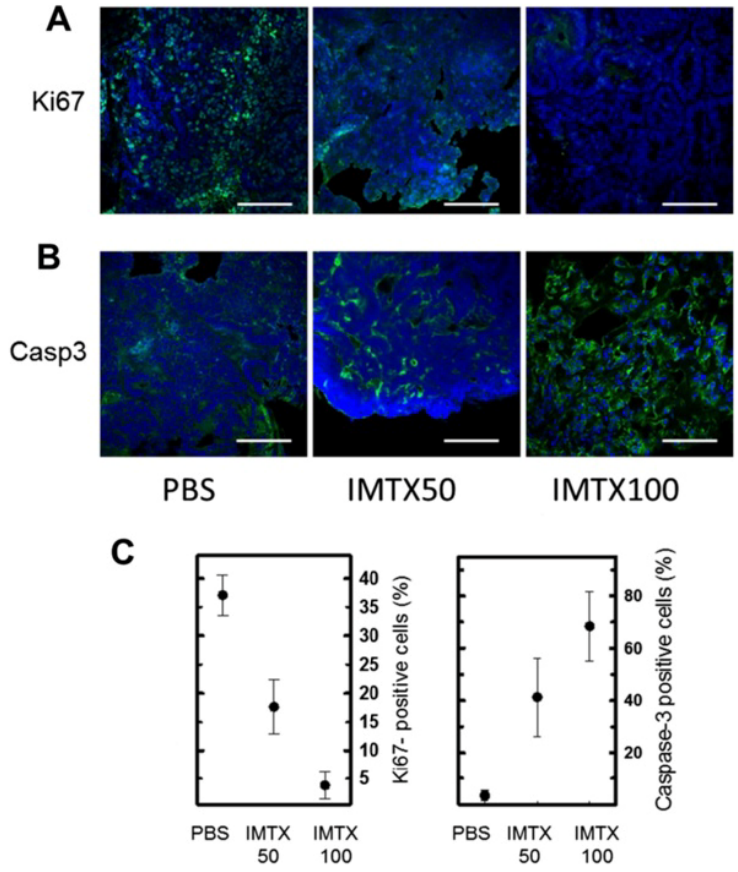

Figure 4 IMTXA33αS-treated tumors show inhibition of cancer cell proliferation and induction of cancer cell apoptosis *in vivo*. **A)** Ki67-positive cells (green) and **B)** active caspase-3-positive cells (green) in tumors. Scale bars: A, 60 μm; B, 60, 30, 40 μm (from left to right). **C)** Quantification of Ki67-positive cells **(A)** and active-caspase-3-positive cells **(B)** in the tumors are shown in the corresponding graphs. Statistical analysis of IMTX50 or IMTX100-treated tumors *vs* vehicle-treated tumors rendered $p < 0.001$ **(A)** and $p < 0.05$ **(B)**.

in the future have to be considered including different via of administration, determination of the maximum tolerated dose of the molecule, limiting toxicities or a more thorough analysis of the effect on the different tissues and organs, among others. However, the results herein presented would be enough and consistent to be considered as a proof of concept.

As mentioned before, other immunoconjugates constructs have been previously used in preclinical or clinical trials based on mAbA33, most of them using the whole antibody molecule conjugated with radionuclides (Scott et al. 2005; Almqvist et al. 2006; Panjideh et al. 2008). In these clinical trials no toxicity in normal colon was observed, although GPA33 could be detected in the colonic epithelium (Scott et al. 2005; Almqvist et al. 2006; Ackerman et al. 2008; Panjideh et al. 2008). The surface persistence of the GPA33 antigen and normal intestinal epithelium shedding contribute to the very good retention in tumor tissue and the rapidly released form normal cells (Almqvist et al. 2006; Ackerman et al. 2008), supporting the selective localization into the tumor of mAbA33 immunoconjugates, as shown by in vivo biodistribution in mice models (Scott et al. 2005; Almqvist et al. 2006; Panjideh et al. 2008). Our results provide further evidence of the potential of GPA33 antigen as a specific tumor-marker for colon cancer becoming a good alternative to those actually used in therapy (Sliwkowski and Mellman 2013). In this sense, biodistribution studies with IMTXA33αS would provide useful information about half-life and localization of the immunotoxin and would be a step further in the thorough characterization of its antitumor action.

On the other hand, RNases from different origins have arisen as new candidates for the toxic domain of immunotoxins versus the commonly used ricin, *Diphtheria* or *Pseudomonas* toxins, to improve their efficacy or to circumvent immunogenicity or side adverse effects (Ardelt 2013; D'Avino et al. 2014). In regard to IMTXA33αS immunogenicity, neither ribotoxins as α-sarcin (Rathore and Batra 1996; Goyal and Batra 2000; Lacadena et al.

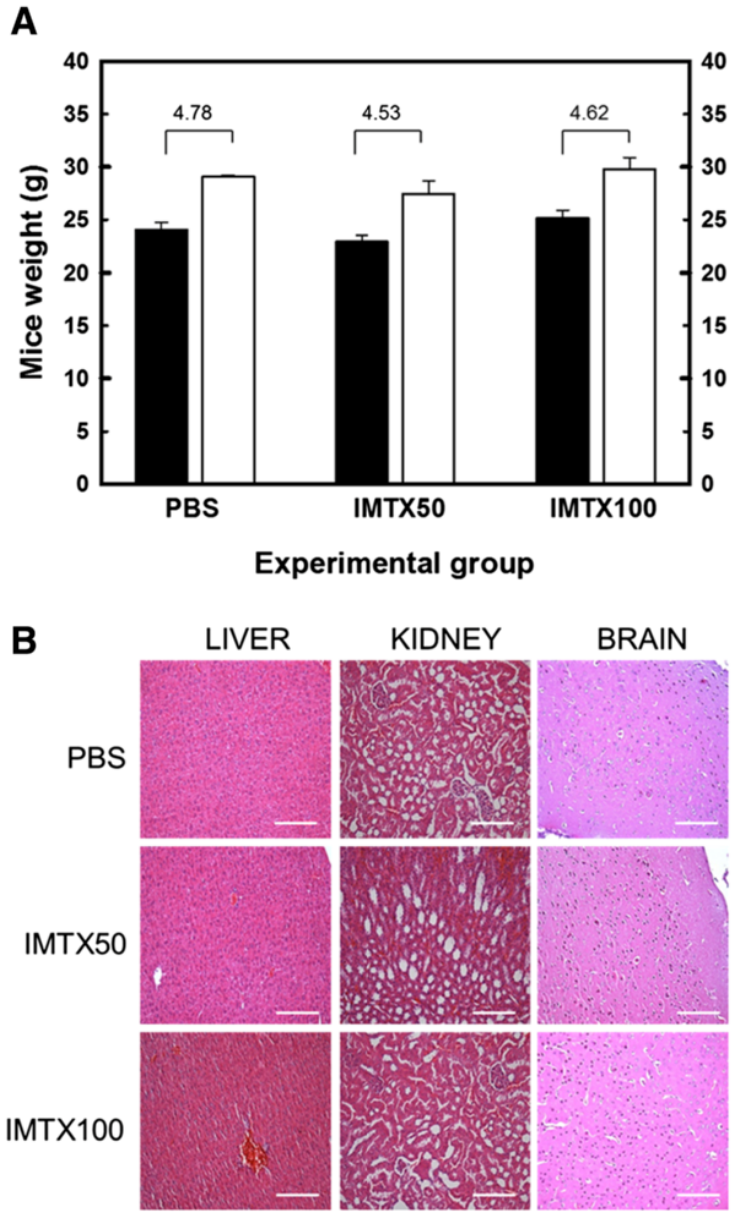

Figure 5 IMTXA33αS-treated mice show no major toxicity effects *in vivo*. A) Mice weight measurement before and after treatment. Black bars (before treatment), white bars (after treatment). Increase in weight (g) is indicated for each experimental group. No significant statistical differences were observed between the different experimental groups. **B)** Hematoxylin & eosin staining of representative tissue sections from non-treated (PBS) or treated- (IMTX50 or IMTX100) mice. Scale bars: 120 μm (liver), 40 μm (kidney) and 100 μm (brain). No significant differences in histological tissue analysis of the selected organs were observed.

2007) nor the humanized scFVA33 domain used (Welt et al. 2003; Scott et al. 2005) would contribute to a potential immune response against the immunotoxin (Carreras-Sangrà et al. 2012), avoiding the inactivation of its antitumor action. This issue should be considered and confirmed when assayed in future clinical trials. In fact, in addition to the ribotoxins advantages mentioned above for their use as immunotoxins toxic moiety, the results show that nonspecific toxicity associated with ribotoxins is extremely reduced when included in an immunotoxin.

As mentioned in the Introduction, different ribotoxins have been used before as components of immunotoxins and assayed in vitro (Orlandi et al. 1988; Wawrzynczak et al. 1991; Better et al. 1992; Rathore et al. 1997; Goyal and Batra 2000). Unfortunately none of the ribotoxin-

based immunotoxins was studied beyond a preliminary characterization, most probably due to their larger size, which could hinder their correct internalization into solid tumors, or to the low structural stability of the immunoconjugates prepared. IMTXA33αS is the first immunotoxin based on ribotoxins and the scFvA33 that has demonstrated its antitumor effectiveness on *in vivo* assays.

Conclusion

IMTXA33αS, an immunotoxin based in ribotoxin α-sarcin, exhibits highly specific antitumor effectiveness in mice harbouring human colon tumor xenografts. It also exhibits anti-angiogenesis, anti-proliferative properties, and lacks adverse side effects. Thus, the results herein presented are a step further in the potential clinical application of IMTXA33αS and, in general, of ribotoxins-based immunotoxins.

Materials and methods

Materials

All chemicals were of molecular biology grade and used without further purification.

Protein production, purification and in vitro characterization

IMTXA33αS was expressed in *Pichia pastoris*. Structural and *in vitro*-functional characterization of the isolated immunotoxin was performed as previously reported (Carreras-Sangrà et al. 2012; Tomé-Amat et al. 2012) before assayed *in vivo*. If needed, the purified immunotoxin was lyophilized and stored at −80°C until use.

Cell line culture

Human colon carcinoma SW1222 and LIM1215 cells were provided by Dr. Carl Batt under the partnership Cornell University-Ludwig Institute of Cancer Research. SW122 cells were used as GPA33-positive cells for xenografts induction. Both cell lines were authenticated in 2010 by short tandem repeat (STR) analysis. Cells were also tested by flow cytometry, using a double labelling of GPA33 and CD44, characteristic of this cell line, immediately before any *in vitro* or *in vivo* assays. Double checking of GPA33 expression was also made by western blot using cellular extracts. Human colon adenocarcinoma HT-29 (ATCC:HTB-38) and epidermoid carcinoma A431 (ATCC:CRL-1555) cells were obtained from the ATCC Cell Biology Collection which carried out its characterization by STR analysis. Cells were grown as described (Carreras-Sangrà et al. 2012; Tomé-Amat et al. 2012) in Dulbecco's modified Eagle's medium (DMEM), containing glutamine (300 μg/ml), 50 U/ml of penicillin, and 50 μg/ml of streptomycin, and supplemented with 10% fetal bovine serum (FBS). Incubation was performed at 37°C in 5% CO_2 humidified atmosphere. Harvesting and propagation of cultures were routinely performed by trypsinization. The number of cells used was determined using a haemocytometer.

In vitro cytotoxicity assay

To evaluate the *in vitro* cytotoxic activity of α-sarcin-based constructions, inhibition of protein biosynthesis was determined as described (Lacadena et al. 2007; Carreras-Sangrà et al. 2012). GPA33-positive cells (SW1222 and LIM1215) and GPA33-negative cells (HT-29 and A431) were incubated with different concentrations of IMTXA33αS, free fungal wild-type α-sarcin or scFvA33. After 72 h of incubation at 37°C, the medium was removed and replaced with a fresh one supplemented with 1 mCi per well of L-[4,5-3H]-Leucine (166 Ci/mmol; Amersham, UK). After an additional incubation for 6 h, the medium was also removed and cells were fixed with 5.0% (w/v) trichloroacetic acid in PBS and washed three times with cold ethanol. The resulted dried pellet was dissolved in 0.2 ml of 0.1 M NaOH containing 0.1% SDS, and radioactivity was counted on a Beckman LS3801 liquid scintillation counter. To calculate the IC_{50} values (protein concentration inhibiting 50% protein synthesis) the results were expressed as a percentage of the radioactivity incorporated in control samples incubated without any of the three proteins, wild type α-sarcin, scFvA33 or the immunotoxin IMTXA33αS. Three independent replicate assays were conducted to calculate the average IC_{50} values.

Animal treatment

All animal procedures were performed with the approval of the Complutense University Animal Experimentation Committee, according to the European official regulations.

Balb/c *nude* male mice (7 weeks old) were purchased from Harlan Laboratories S.A. (Barcelona, Spain) to evaluate the *in vivo* effect of IMTXA33αS against colorectal cancer induced xenografts. Two sets of assays were performed, using the Animal Facilities of either the Complutense University or the Centro Investigaciones Biológicas-Consejo Superior Investigaciones Biológicas (CIB-CSIC) in Madrid.

Mice were allocated into three experimental groups (n = 8): PBS (phosphate buffered saline), IMTX50 and IMTX100 (treatment with 50 or 100 μg of immunotoxin per injection, respectively). Prior to the experiments, animals were given a 7-day adaptation period with free access to food and water at all times. Each mouse received a subcutaneous injection into the right flank of 2×10^6 SW1222 cells, resuspended in 100 μl PBS and 100 μl of Matrigel (BD Biosciences). Once the

tumor volume raised 50–100 mm^3, mice were injected intraperitonealy either with PBS or immunotoxin. Seven doses, every 48 hours, of PBS or the two different amounts of immunotoxin (50 or 100 μg) were given. Two control groups, free-α-sarcin wild type (n = 3) and scFvA33 (n = 3) were also included. In this case, we used doses of 40 and 61 μg per injection, respectively, which were equivalent to the highest dose of immunotoxin used (IMTX100), (2.2 nanomoles),

Tumors were routinely measured during this period with an external caliper, and volume was calculated as $(width/2)^2$ x (length/2). Mice were also weighted throughout the experiment. At the end of the treatment, animals were sacrificed and tumors and organs were collected. Tumors were divided in portions and stored fixed in buffered 4% paraformaldehyde (PFA) or in Tissue-Tek (Sakura Finetek Europe) for immunofluorescence staining.

ImmunofluorescenceTumor analysis

Histological analyses of excised tumors were performed. Tissue-teK embedded tumor sections of 8–10 μM thickness were obtained using a cryotome (Criocut Leica CM), fixed in methanol, washed with IFF buffer (5% FBS, 1% BSA, PBS) and incubated with anti-GPA33 polyclonal human mAb A33 (Santa Cruz Biotechnologies), anti-CD31 (Pharmingen/BD Biosciences), anti-Ki67 (Thermo Scientifics) or anti-Caspase3 (Cell Signaling Technology) antibodies. CD31, Ki67 and active-caspase3 are well-stablished markers for angiogenesis, proliferation and apoptosis, respectively. Secondary anti-rabbit antibodies, labelled with AlexaFluor 647 for GPA33 and Ki67 detection or anti-mouse AlexaFluor 488 for CD31 and Caspase3, were purchased from Invitrogen. Cell nuclei were stained with Prolong Gold with 40,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). All these incubations were performed at room temperature. A Leica TCS P2 confocal microscope and the corresponding LCS lite software were used to obtain the fluorescence images. At least three sections of two representative tumors from each experimental group were used.

Histopathological analysis

Brain, spleen, liver, kidneys, lungs and intestine were collected at the end of the treatment and fixed in 4% PFA. Collected organs were visually analysed for possible macroscopic changes resulting from treatment. Histological assessment of organs samples was performed by hematoxylin and eosin (H&E) staining. The samples were sliced into sections as described above, fixed in PFA and stained for H&E analysis. Morphological changes were evaluated using a light microscope and H&E-stained images were acquired.

Statistical analysis

ANOVA with a post hoc analysis by the Student-Newman-Keuls' test was used to compare variations in the mean tumor sizes at different treatment time points in each experimental group. Differences between experimental groups were considered statistically significant at $P < 0.05$. All values were expressed as arithmetic means ± sem (standard error of the media). For immunofluorescence tumor statistical analysis, at least three sections of two representative tumors from each experimental group were used.

Abbreviations

ADEPT: Antibody-directed-enzyme-produg-therapy; DAPI: 40,6-diamidino-2-phenylindole; DMEM: Dulbecco's modified Eagle's medium; EGFR: Epidermal growth factor receptor; FBS: Fetal bovine serum; GPA33: Glycoprotein A33; GRAS: Generally regarded as safe; H&E: Hematoxylin and eosin; huA33: A33 humanized monoclonal antibody; JAM: Junction adhesion molecules; PBS: Phosphate buffered saline, PFA: Paraformaldehyde; RNases: Ribonucleases; scFv: Single-chain variable fragments; SRL: Sarcin/ricin loop; STR: Short tandem repeat; VEGF: Vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JTA planned and performed experiments, analyzed data and drafted the manuscript. MO carried out the *in vivo* assays and analyzed data. JRH carried out the *in vitro* and *in vivo* assays, analyzed data and performed the statistical analysis. EPG and CA participated in the design and carried out the *in vivo* assays. CS participated in the design of the in vivo assays, analyzed data and helped to draft the manuscript. LM carried out the histopatologic studies. AMP participated in the design of experiments, analyzed data and drafted the manuscript. JGG analyzed data and helped to draft the manuscript. JL planned and performed experiments, analyzed data, performed statistical analysis and coordinated and drafted the manuscript. All authors read and approved the final manuscript.

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References

Ackerman ME, Chalouni C, Schmidt MM, Raman VV, Ritter G, Old LJ, Mellman I, Wittrup KD (2008) A33 antigen displays persistent surface expression. Cancer Immunol Immunother 57:1017–1027

Almqvist Y, Steffen AC, Tolmachev V, Divgi CR, Sundin A (2006) In vitro and in vivo characterization of ^{177}Lu-huA33. A radioimmunoconjugate against colorectal cancer. Nuclear Med Biol 33:991–998

Ardelt W (2013) ImmunoRNase fusions. In Fusion Protein Technologies for Biopharmaceuticals: Applications and Challenges, First Edition. Edited by Stefan R. Schmidt. John Wiley & Sons, Inc. Published by John Wiley & Sons, Inc. pp.337-353

Barendswaard EC, Humm JL, O'Donoghue JA, Sgouros G, Finn RD, Scott AM, Larson SM, Welt S (2001) Relative Therapeutic Efficacy of ^{125}I- and ^{131}I Labeled Monoclonal Antibody A33 in a Human Colon Cancer Xenograft. J Nuclear Med 42:1251–1256

Better M, Bernhard SL, Lei SP, Fishwild DM, Carroll SF (1992) Activity of recombinant mitogillin and mitogillin immunoconjugates. J Biol Chem 267:16712–16718

Carreras-Sangrà N, Álvarez-García E, Herrero-Galán E, Tomé J, Lacadena J, Alegre-Cebollada J, Oñaderra M, Gavilanes JG, Martínez-Del-Pozo A (2008) The therapeutic potential of fungal ribotoxins. Curr Pharm Biotechnol 9:153–160

Carreras-Sangrà N, Tomé-Amat J, García-Ortega L, Batt CA, Oñaderra M et al (2012) Production and characterization of a colon cancer specific immunotoxin based on the fungal ribotoxin α-sarcin. Prot Eng Des Sel 25:425–435

Coelho V, Demedde J, Petrausch U, Panjideh H, Fuchs H, Menzel C, Dübel S, Kellholz U, Thiel E, Deckert PM (2007) Design, construction, and in vitro analysis of A33scFv::CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy in colon cancer. Int J Oncol 31:951–957

D'Avino C, Paciello R, Riccio G, Coppola M, Laccetti P, Maurea N, Raines RT, De Lorenzo C (2014) Effects of a second-generation human anti-ErbB2 ImmunoRNase on trastuzumab-resistant tumors and cardiac cells. Prot Eng Des Sel 27:83–88

Dienstmann R, Markman B, Tabernero J (2012) Application of monoclonal antibodies as cancer therapy in solid tumors. Curr Clin Pharmacol 7:137–145

Dougan M, Dranoff G (2009) Immune therapy for cancer. Annu Rev Immunol 27:83–117

Eng C (2010) The evolving role of monoclonal antibodies in colorectal cancer: Early presumptions and impact on clinical trial development. Oncologist 15:73–84

Frankel AE, Ramage J, Kiser M, Alexander R, Kucera G, Miller MS (2000) Characterization of diphtheria fusion proteins targeted to the interleukin-3 receptor. Protein Eng 13:575–581

García-Ortega L, Álvarez-García E, Gavilanes JG, Martínez-del-Pozo A, Joseph S (2010) Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. Nucleic Acids Res 38:4108–4119

Gehlsen KR, Gong R, Bramhill D, Wiersma DA, Kirkpatrick SA, Wang Y, Feng Y, Dimitrov DS (2012) Pharmacokinetics of engineered human monomeric and dimeric CH2 domains. mAbs 4:466–474

Goetsch L, Haeuw JF, Beau-Larvor C, Gonzalez A, Zanna L, Malissard M, Lepecquet AM, Robert A, Bailly C, Broussas M, Corvaia N (2013) A novel role for junctional adhesion molecule-A in tumor proliferation: Modulation by an anti-JAM-A monoclonal antibody. Int J Cancer 132:1463–1474

Goldin A, Serpick AA, Mantel N (1966) Experimental screening procedures and clinical predictability value. Cancer Chemother Rep 50:173–218

Goyal A, Batra JK (2000) Inclusion of a furin-sensitive spacer enhances the cytotoxicity of ribotoxins restrictocin containing recombinant single-chain immunotoxin. Biochem J 345:247–254

Heath JK, White SJ, Johnstone CN, Catimel B, Simpson RJ, Moritz RL, Tu GF, Ji H, Whitehead RH, Groenen LC, Scott AM, Ritter G, Cohen L, Welt S, Old LJ, Nice EC, Burgess AW (1997) The human A33 antigen is a transmembrane glycoprotein and a novel member of the immunoglobulin superfamily. Proc Natl Acad Sci U S A 94:469–474

Jain RK (2001) Delivery of molecular medicine to solid tumors: lessons from in vivo imaging of gene expression and function. J Control Release 74:7–25

Kirui DK, Khalidov I, Wang Y, Batt CA (2013) Targeted near-IR hybrid magnetic nanoparticles for in vivo cancer therapy and imaging. Nanomed Nanotechnol Biol Med 9:702–711

Kuespert K, Pils F, Hauck CR (2006) CEACAMs: their role in physiology and pathophysiology. Curr Opin Cell Biol 18:565–571

Lacadena J, Martínez-del-Pozo A, Martínez-Ruiz A, Pérez-Cañadillas JM, Bruix M, Mancheño JM, Oñaderra M, Gavilanes JG (1999) Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin α-sarcin. Proteins 37:474–484

Lacadena J, Álvarez-García E, Carreras-Sangrà N, Herrero-Galán E, Alegre-Cebollada J, García-Ortega L, Oñaderra M, Gavilanes JG, Martínez del Pozo A (2007) Fungal ribotoxins:molecular dissection of a family of natural killers. FEMS Microbiol Rev 31:212–237

Madhumati J, Verma RS (2012) Therapeutic targets and recent advances in protein immunotoxins. Curr Opin Microbiol 15:300–309

McSherry EA, McGee SF, Jirstrom K, Doyle EM, Brennan DJ, Landberg G, Dervan PA, Hopkins AM, Gallagher WM (2009) JAM-A expression positively correlates with poor prognosis in breast cancer patients. Int J Cancer 125:1343–1351

Olmo N, Turnay J, González de Buitrago G, López de Silanes I, Gavilanes JG, Lizarbe MA (2001) Cytotoxic mechanism of the ribotoxin α-sarcin. Induction of cell death via apoptosis. Eur J Biochem 268:2113–2123

Olombrada M, Rodríguez-Mateos M, Prieto D, Pla J, Remacha M, Martínez-del-Pozo A, Gavilanes JG, Ballesta JP, García-Ortega L (2014a) The acidic ribosomal stalk proteins are not required for the highly specific inactivation exerted by α-sarcin of the eukaryotic ribosome. Biochemistry 53:1545–1547

Olombrada M, Martínez-del-Pozo A, Medina P, Budia F, Gavilanes JG, García-Ortega L (2014b) Fungal ribotoxins: Natural protein-based weapons against insects. Toxicon 83:69–74

Onda M, Beers R, Xiang L, Lee B, Weldon JE, Kreitman RJ, Pastan I (2011) Recombinant immunotoxin against B-cell malignancies with no immunogenicity in mice by removal of B-cell epitopes. Proc Natl Acad Sci U S A 108:5742–5747

Orlandi R, Canevari S, Conde FP, Leoni F, Mezzanzanica D, Riparmonti M, Colnaghi MI (1988) Immunoconjugate generation between the ribosome inactivating protein restrictocin and an anti-human breast carcinoma MAB. Cancer Immunol Immunother 26:114–120

Panjideh H, Coelho VC, Demedde J, Bachran C, Föster G, Franke J, Fasold P, Fuchs H, Thiel E, Deckert PM (2008) Biodistribution and efficacy of [^{131}I]A33scFv::CDy, a recombinant antibody-enzyme protein for colon cancer. Int J Oncol 32:925–930

Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ (2007) Immunotoxin treatment of cancer. Annu Rev Med 58:221–237

Rathore D, Batra JK (1996) Generation of active immunotoxins containing recombinant restrictocin. Biochem Biophys Res Commun 222:58–63

Rathore D, Nayak SK, Batra JK (1997) Overproduction of fungal ribotoxin α-sarcin in Escherichia coli: generation of an active immunotoxin. Gene 190:31–35

Roga V, Hedeman LP, Olson BH (1971) Evaluation of mitogillin (NSC-69529) in the treatment of naturally ocurrin canine neoplasms. Cancer Chemother Rep 55:101–113

Sapra P, Shor B (2013) Monoclonal antibody-based therapies in cancer: Advances and challenges. Pharmacol Ther 138:452–469

Schindler DG, Davies JE (1977) Specific cleavage of ribosomal RNA caused by alpha sarcin. Nucleic Acids Res 4:1097–1100

Schindler J, Sausville E, Messmann R, Uhr JW, Vitetta ES (2001) The toxicity of deglycosylated ricin A chain-containing immunotoxins in patients with Non-Hodgkin's is exacerbated by prior radiotherapy: A retrospective analysis of patients in five clinical trials. Clin Cancer Res 7:255–558

Scott AM, Lee FT, Jones R, Hopkins W, MacGregor D, Cebon JS, Hannah A, Chong GUP, Papenfuss A, Rigopoulos A, Sturrock S, Murphy R, Wirth V, Murone C, Smyth FE, Knight S, Welt S, Ritter G, Richards E, Nice EC, Burgess AW, Old LJ (2005) A phase I trial of humanized monoclonal antibody A33 in patients with colorectal carcinoma: Biodistribution, pharmacokinetics, and quantitative tumor uptake. Clin Cancer Res 11:4810–4817

Sliwkowski MX, Mellman I (2013) Antibody therapeutics in cancer. Science 341:1192–1198

Tol MD, Punt CDJ (2010) Monoclonal antibodies in the treatment of metastatic colorectal cancer: A Review. Clin Ther 32:437–453

Tomé-Amat J, Menéndez-Méndez A, García-Ortega L, Batt C, Oñaderra M, Martínez-del-Pozo A, Gavilanes JG, Lacadena J (2012) Production and characterization of scFvA33T1, an immunotoxin targeting colon cancer cells. FEBS J 279:3022–3032

Wawrzynczak EJ, Henry RV, Cumber AJ, Parnell GD, Derbyshire EJ, Ulbrich N (1991) Biochemical, cytotoxic and pharmacokinetic properties of an immunotoxin composed of a mouse monoclonal antibody Fib75 and the ribosome-inactivating protein alpha-sarcin from *Aspergillus giganteus*. Eur J Biochem 196:203–209

Welt S, Ritter G, Williams C Jr, Cohen LS, John M, Jungbluth A, Richards EA, Old LJ, Kemeny NE (2003) Phase I Study of anticolon cancer humanized antibody A33. Clin Cancer Res 9:1338–1346